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Express Mail No.:

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Hong Jin *et al.*

Confirmation No.: 8169

Application No.: 09/724,388

Group Art Unit: 1648

Filed: November 28, 2000

Examiner: Lucas, Zachariah

For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES Attorney Docket No.: 7682-051-999

DECLARATION OF DAVID K. CLARKE  
UNDER 37 C.F.R. § 1.131

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

I, DAVID K. CLARKE, who resides at 3205 Whispering Hills, Chester, New York 10918, do declare that:

1. I am a co-inventor of the invention described in the above-identified application and as claimed in pending claims 7 to 21 (annexed hereto as Exhibit A1).

2. The above-identified application and pending claims relate to genetically manipulated, infectious, replication-competent, non-segmented, negative-strand RNA viruses wherein the viral genome has been modified, *e.g.*, the genome contains deletions or insertions. The invention and pending claims encompass genetically manipulated paramyxoviruses in which the viral genome has been modified to result in a packaged infectious virion with an attenuated phenotype, *i.e.*, the virus is able to go through only one or a few rounds of replication in the host. The invention encompasses genetically manipulated RSV, vaccines comprising genetically manipulated RSV and methods for generating genetically manipulated RSV. The application claims priority to a series of



applications including earlier filed application no. 09/161,122 filed September 25, 1998, which, in turn, is a continuation-in-part of our earlier application no. 08/316,439, filed September 30, 1994 (Grand-Parent Application, attached hereto as Exhibit A2, now U.S. Patent No. 5,840,520, issued November 24, 1998).

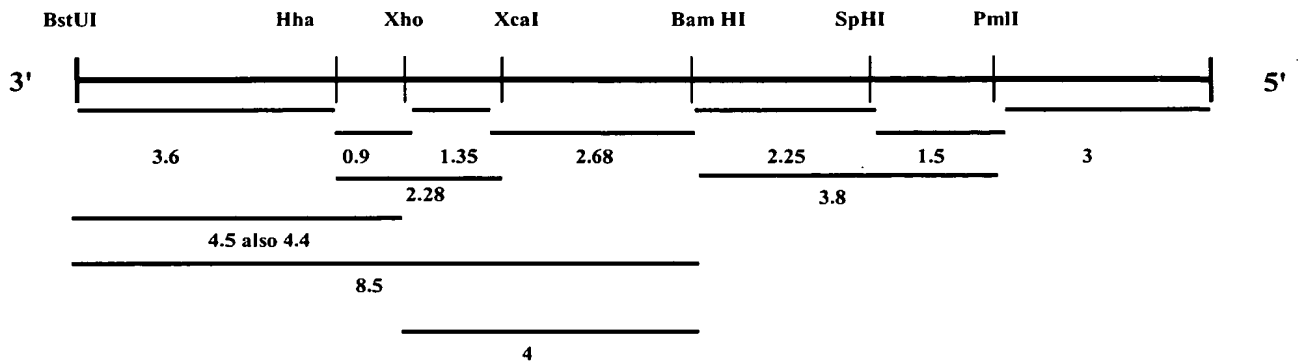
3. Annexed hereto as Exhibit B is a copy of a page from my laboratory notebook describing a research plan to genetically manipulate the genome of RSV to result in a virion with an attenuated phenotype. Such genetic manipulations include deletions and insertions of sequences in the viral genome. Although the date on the copy has been redacted, the notebook page is dated prior to January 10, 1994. Thus, demonstrating that prior to January 10, 1994, I had conceived a genetically manipulated, replication-competent, infectious, non-segmented negative strand RNA virus having an attenuated phenotype as described in the Grand Parent application and described and claimed in the instant application.

4. Annexed hereto as Exhibits C through E are copies of pages from my laboratory notebooks that demonstrate that I or person(s) acting under my direction and supervision worked with diligence from the conception of the invention up to the filing date of the Grand Parent application to reduce the claimed invention to practice. This is evidenced by the facts described below and by the attached exhibits.

5. Exhibits C to E are true copies of my laboratory notebooks that describe experiments relating to, among others, the generation of a full length cDNA encoding the genome of RSV. The pages of the laboratory notebooks are numbered consecutively for ease of reference. The references to pages in the laboratory notebooks are

to the production numbers stamped at the bottom right corner of the pages in the notebooks. The dates in the laboratory notebooks have been redacted. Further, references to events that would lead to the date of the entry into the laboratory notebook have also been redacted. The laboratory notebooks document experiments that were conducted during the time period from briefly before the date of the conception document until the filing date of the Grand Parent application.

6. In particular, the laboratory notebooks document the isolation of genomic RSV RNA, RT-PCR amplification of fragments of the RSV genome, optimization of growth cultures of RSV, the subcloning of the RT-PCR fragments of the RSV genome in different cloning vectors and the ligation of the different RT-PCR fragments to obtain a cDNA encoding the full length genome of RSV. The fragments of the RSV genome that are mentioned in the laboratory notebooks are set forth in the following genomic restriction map of RSV. Restriction enzyme sites are shown on above the line representing the genome and the numbers below the line refer to the approximate sizes of the fragments in kilobasepairs. In this declaration, DNA fragments are referred to by the name of the flanking restriction sites followed by the approximate length of the fragment. For example, the first fragment at the 3' end of the virus is designated BstU/Hha-3.5. Synonyms of the DNA fragments are listed in the respective paragraphs that describe the work relating to the different DNA fragments. The map below also shows how the different DNA fragments were to be assembled to obtain the full length RSV cDNA.



Genomic Restriction Map of RSV

7. The following paragraphs explain the type of experiments to obtain the different DNA fragments shown in the diagram above and the experiments to assemble these fragments into a full-length RSV cDNA; references in the following paragraphs are to production numbers in Exhibits C to E. In general, the entries in connection with obtaining the DNA fragments relate to RT-PCR amplification of the fragment, purification of the amplified DNA material, ligation into a cloning vector, transfection into bacteria, preparation of plasmid DNA from the transfected bacteria, and restriction analysis of the plasmid DNA.

7a. Work relating to the growth of RSV, infection of different cell types with RSV, the harvest and purification of virus, and the purification of genomic RNA of RSV are described at production nos. 47-65, 67-70, 72-79, 81, 95, 105, 110, 113, 117, 121-125, 127, 133, 137-144, 146, 148, 150-151, 153, 155, 169, 176-177, 188, 193, 212, 249, 269, 322, and 353-354 of my laboratory notebooks. These entries also relate to obtaining RS virus stocks from different sources, the optimization of growth conditions for RSV, testing of different cell lines for virus replication, optimization of virus preparation, and the purification of genomic RNA from the virus.

7b. Work relating to the performance of RT-PCR to obtain DNA fragments that encode parts of the viral genome are described at production nos. 65-66, 83-84, 87, 89, 90, 157, 160-165, 170, 172, 173, 179-180, 205, 242, 246-247, 282, and 393-394 of my laboratory notebooks. These entries also relate to obtaining the reagents and biochemicals for conducting RT-PCR, designing of primers, designing of cloning strategies, and the optimization of the conditions for the performance of RT-PCR. Work relating to the preparation of cloning vectors for subcloning of the RT-PCR fragments is described at production nos. 115-116 (puc19),

7c. Work relating to the RT-PCR amplification and the subcloning of the BstU/Hha-3.6 fragment (also referred to as "BstU/Hha" and "3.5K" and "3.6K" and "3.7K") is described at production nos. 264, 270, 272, 274, 276-277, 279, 281, 298, 301-302, 322-323, 325, 328, 339, 343, 367, 369-371, 378, and 381-382 of my laboratory notebooks. These entries also relate to large scale plasmid preparation of the cloned fragment.

7d. Work relating to the RT-PCR amplification and the subcloning of the Hha/Xho-0.9 fragment (also referred to as "0.9K" and "H/Xho" and "Hha/Xho" and "clone 8") is described at production nos. 327-331, 336-337, 345-350, 352, 361-362, 366-368, 371, 376, 378-380, 382, 386-387, and 392 of my laboratory notebooks. These entries also relate to the development of alternative cloning strategies, and the evaluation of restriction analysis.

7e. Work relating to the RT-PCR amplification and the subcloning of the Xho/Xca-1.35 fragment (also referred to as "1.3K" (370) and "Xho/Xca" and "clone 2") is described at production nos. 295-298, 299, 301, 331, 336-337, 345-350, 352, 358-368, 370, 374, 377-380, 382, 386-387, 393, 399, 423, 426-427, 429-432, 434, 459-461, 465-467, 471-

473, 475-476, and 481-487 of my laboratory notebooks. These entries also relate to the development of alternative cloning strategies, and the evaluation of restriction analysis.

7f. Work relating to the RT-PCR amplification and the subcloning of the Xca/BamHI-2.68 fragment (also referred to as "Xca/Bam" and "Bam/Xca" and "X/B" and "2.6K" and "2.7K") is described at production nos. 261-262, 264, 268, 270-271, 274-275, 306, 322, 343, 369-370, 373-374, and 377-378 of my laboratory notebooks.

7g. Work relating to the RT-PCR amplification and the subcloning of the BamHI/SpHI-2.25 fragment (also referred to as "BamHI/SphI" and "Bam/Sph" and "2.25Kb" or "2.25K") is described at production nos. 258-260, 268, 270-271, 274-275, 305-306, 343, 354-357, 407-411, 433, 457, 459, 461-464, 472, 475, 480, 484, and 486-490 of my laboratory notebooks. These entries also relate to restriction mapping of the BamHI/SpHI-2.25 fragment.

7h. Work relating to the RT-PCR amplification and the subcloning of the SpHI/PmlI-1.5 fragment (also referred to as "1.5K") is described at production nos. 250-254, 256-257, 259-260, 263, 266-267, 305, 343, 354-357, 409-410, 412, 418-420, 422-423, 425-434, 457, 461-464, 486, and 488 of my laboratory notebooks. These entries also relate to restriction mapping of the SpHI/PmlI-1.5 fragment.

7i. Work relating to the RT-PCR amplification and the subcloning of the PmlI/5'end-3 fragment (also referred to as "3K" and "3K sure") is described at production nos. 179-180, 189-190, 193-196, 198, 298, 301-303, 309, 311, 316, 343, 407-409, 411-412,

418-424, 433, and 457 of my laboratory notebooks. These entries also relate to large scale plasmid preparation and the restriction analysis of the plasmid DNA.

7j. Work relating to the RT-PCR amplification and subcloning of the BamHI/PmlI-3.8 fragment (also referred to as "3.8Kb") is described at production nos. 181, 185, 194, 196, 197, 201-202, 204, 207-210, 214-216, 240-242, and 243 of my laboratory notebooks. These entries also relate to designing of primers to amplify the BamHI/PmlI-3.7 fragment.

7k. Work relating to the RT-PCR amplification and subcloning of the HhaI/XcaI-2.28 fragment (also referred to "2.3k" and "Hha/Xca" and "H/Xca" and "Sure 2.3" and "H/X") is described at production nos. 261-262, 268, 275-281, 285, 287-294, 299-300, 305, 307, 309, 311-321, 324, 327-330, 332, 334-336, 337, 339-345, 372-374, and 388-391 of my laboratory notebooks. These entries also relate to designing of primers to amplify the HhaI/XcaI-2.28 fragment and the development of alternative cloning strategies, such as the use of different competent bacterial cells for the propagation of the plasmid DNA.

7l. Work relating to the RT-PCR amplification and subcloning of the BstUI/XhoI-4.5 fragment (this fragment is also referred to as "4.4K") is described at production nos. 325-326, 414-417, and 421 of my laboratory notebooks.

7m. Work relating to the RT-PCR amplification and subcloning of the Xho/BamHI-4 fragment (also referred to as "4K" and "Xho/Bam") is described at production nos. 205-207, 209-211, 214, 379, and 383-384 of my laboratory notebooks.

7n. Work relating to the generation of the leader and trailer regions of the RSV is described at production nos. 92-94, 113-114, 127, 129-136, 138, 139, 283-284, and 405 of my laboratory notebooks.

7o. Work relating to the ligation of the BstU/Hha-3.6 fragment, the HhaI/XcaI-2.28, and the Xca/BamHI-2.68 fragment to result in BstUI/BamHI-8.5 are described at production nos. 347-352 of my laboratory notebooks.

7p. Work relating to the ligation of the BstU/Hha-3.6 fragment and the Hha/Xho-0.9 fragment to obtain a the BstU/Xho-4.4 fragment (also referred to as "4.4k") is described at production nos. 391-392, 395-398, 400-401, 403, 406, 413, 469, 471, 473, 476-482, 484-485, 489-490 of my laboratory notebooks.

7q. Work relating to the ligation of the BstU/Xho-4.4 fragment and the Xho/Xca-1.35 fragment is described at production nos. 474 and 485 of my laboratory notebooks.

7r. Work relating to the ligation of two or three of the BamHI/SpHI-2.25 fragment, the SpHI/PmlI-1.5 fragment, and the PmlI/5'end-3 fragment to each other is described at production nos. 425, 458, 465-466, 468-473, 475, and 491 of my laboratory notebooks.

8. The fragments that are described in paragraphs 7a to 7s were later used to assemble full-length cDNA that encodes the genome of an RSV, which was used to rescue an infectious and replication-competent RSV. The full-length cDNA was genetically

manipulated to generate RSV cDNAs with deletions and insertions, which was used to rescue an infectious and replication-competent RSV with an insertion and/or deletion.

9. Exhibit F is a summary of the work described in the laboratory notebooks that are submitted herewith as Exhibits C to E. Each row in Exhibit F represents one day or, where the experiment was performed over several days, a row represents the corresponding period of time.

10. I declare that since the date of conception of the invention I was diligently working towards the reduction to practice of the invention. The only interruption of my work toward the reduction to practice in the laboratory of more than one week was during the two weeks immediately prior to filing of the Grand Parent application. However, during my absence from the laboratory, my technician, Lisa Paganini, was working on the ligation of the SphI/PmlI-1.5 kb fragment and the BamHI/SphI-2.25 kb fragments to each other in accordance with my instructions as indicated at production no. 491.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: March 24<sup>th</sup> 2004

David K. Clarke  
DAVID K. CLARKE



**EXHIBIT A1: COPY OF PENDING CLAIMS IN U.S. APPLICATION NO. 09/724,388**

**AS OF MARCH 24, 2004**

7. (Currently amended) A genetically manipulated, replication competent, infectious virus of the paramyxoviridae family wherein the virus genome comprises a modification wherein the modification is selected from an insertion, ~~substitution~~ or deletion.

8. (Previously presented) The genetically manipulated, infectious virus of Claim 7, the genome of which has been modified to encode a heterologous sequence.

9. (Withdrawn) The virus of Claim 7 or 8 wherein the virus is a parainfluenza virus.

10. (Previously presented) The virus of Claim 7 or 8 wherein the virus is a respiratory syncytial virus.

11. (Currently amended) A genetically manipulated, replication competent, infectious, non-segmented, negative-stranded RNA virus wherein the virus genome comprises a modification wherein the modification is selected from an insertion, ~~substitution~~ or deletion.

12. (Previously presented) A vaccine comprising the genetically manipulated viruses of Claims 7, 8, or 11 and a pharmaceutically acceptable carrier.

13. (Withdrawn) A method for rescuing a genetically manipulated non-segmented negative-stranded RNA virus comprising

introducing into a host cell expressing a heterologous RNA polymerase:

- (a) one or more DNA molecules encoding the virus N, P and L proteins operably linked to a polymerase binding site;

- (b) a DNA molecule comprising the cDNA of the non-segmented negative stranded RNA virus wherein the cDNA encodes the entire genome of the virus or is modified by the incorporation of a mutation or a heterologous sequence, and wherein the cDNA is transcribed by a heterologous RNA polymerase; and  
isolating the virus produced by the cell.

14. (Withdrawn) The method of Claim 13 wherein the virus is from the paramyxoviridae family.

15. (Withdrawn) The method of Claim 14 wherein the virus is parainfluenza virus.

16. (Withdrawn) The method of Claim 14 wherein the virus is a respiratory syncytial virus.

17. (New) The virus of claim 8, wherein the heterologous sequence is a non-RSV sequence.

18. (New) A genetically manipulated, replication-competent, infectious non-segmented, negative strand RNA virus wherein the virus genome comprises a modification wherein the modification is selected from an insertion, substitution, or deletion of an open reading frame encoding a viral gene product.

19. (New) The virus of claim 18, wherein the virus is from the paramyxoviridae family.

20. (New) The virus of claim 18, wherein the virus is RSV.

21. (New) The virus of claim 18, wherein the open reading frame is the M-2 open reading frame.

**United States Patent** [19]**Clarke et al.**[11] **Patent Number:** **5,840,520**[45] **Date of Patent:** **\*Nov. 24, 1998**[54] **RECOMBINANT NEGATIVE STRAND RNA VIRUS EXPRESSION SYSTEMS**[75] Inventors: **David Kirkwood Clarke**, Pacifica, Calif.; **Peter M. Palese**, Leonia, N.J.[73] Assignee: **Aviron**, Mountain View, Calif.

[\*] Notice: The term of this patent shall not extend beyond the expiration date of Pat. No. 5,166,057.

[21] Appl. No.: **316,439**[22] Filed: **Sep. 30, 1994****Related U.S. Application Data**

[63] Continuation-in-part of Ser. No. 190,698, Feb. 1, 1994, abandoned, which is a continuation of Ser. No. 925,061, Aug. 4, 1992, abandoned, which is a division of Ser. No. 527,237, May 22, 1990, Pat. No. 5,166,057, which is a continuation-in-part of Ser. No. 440,053, Nov. 21, 1989, abandoned, and Ser. No. 399,728, Aug. 28, 1989, abandoned.

[51] Int. Cl.<sup>6</sup> ..... **C12P 21/06; C12P 19/34; A61K 39/12**[52] U.S. Cl. .... **435/69.1; 435/91; 435/235.1; 435/320.1; 424/199.1; 536/23.1**[58] Field of Search ..... **435/69.1, 91, 172.3, 435/235.1, 320.1; 536/23.1; 424/199.1**[56] **References Cited****U.S. PATENT DOCUMENTS**

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(List continued on next page.)

**Primary Examiner**—Michael P. Woodward**Assistant Examiner**—Ali R. Salimi**Attorney, Agent, or Firm**—Pennie & Edmonds LLP

[57]

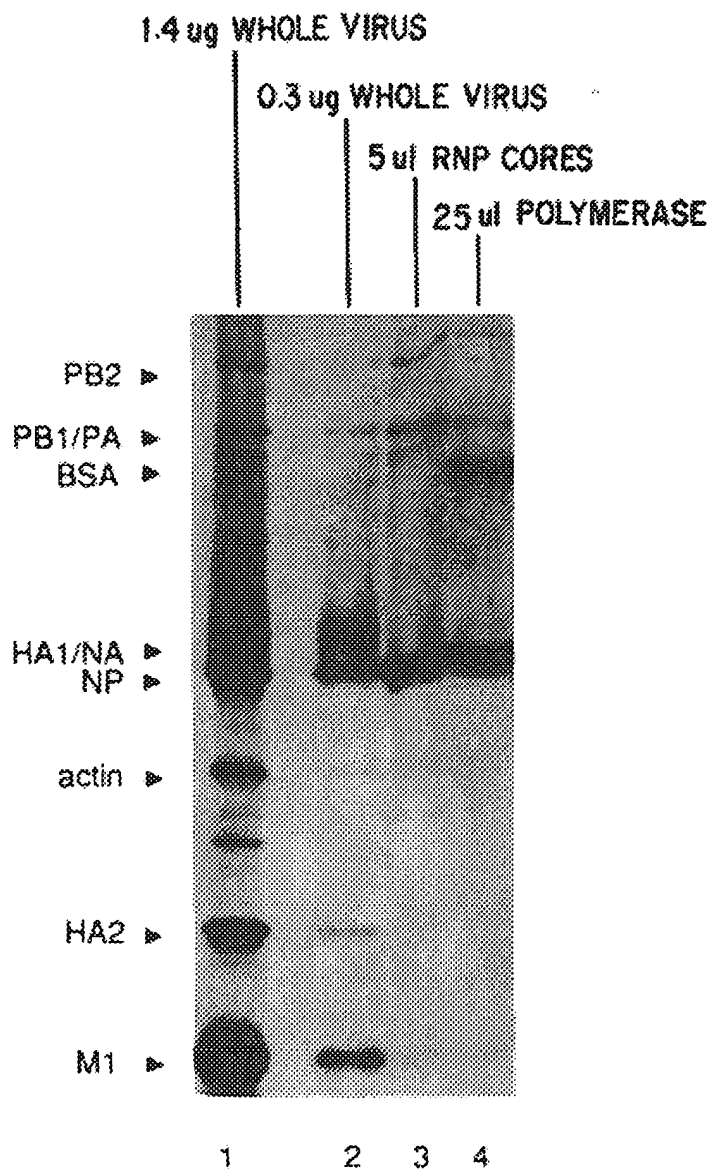
**ABSTRACT**

Recombinant negative strand virus RNA templates which may be used to express heterologous gene products and/or to construct chimeric viruses are described. Influenza viral polymerase, which was prepared depleted of viral RNA, was used to copy small RNA templates prepared from plasmid-encoded sequences. Template constructions containing only the 3' end of genomic RNA were shown to be efficiently copied, indicative that the promoter lay solely within the 15 nucleotide 3' terminus. Sequences not specific for the influenza viral termini were not copied, and, surprisingly, RNAs containing termini identical to those from plus sense cRNA were copied at low levels. The specificity for recognition of the virus-sense promoter was further defined by site-specific mutagenesis. It was also found that increased level of viral protein were required in order to catalyze both the capendonuclease primed and primer-free RNA synthesis from these model templates as well as from genomic length RNAs. This indicated that this reconstituted system had catalytic properties very similar to those of native viral RNPs. High levels of expression of a heterologous gene was obtained using the constructs and methods described. The system was exemplified using Influenza and respiratory syncytial virus.

**9 Claims, 23 Drawing Sheets**

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**FIG. 1**

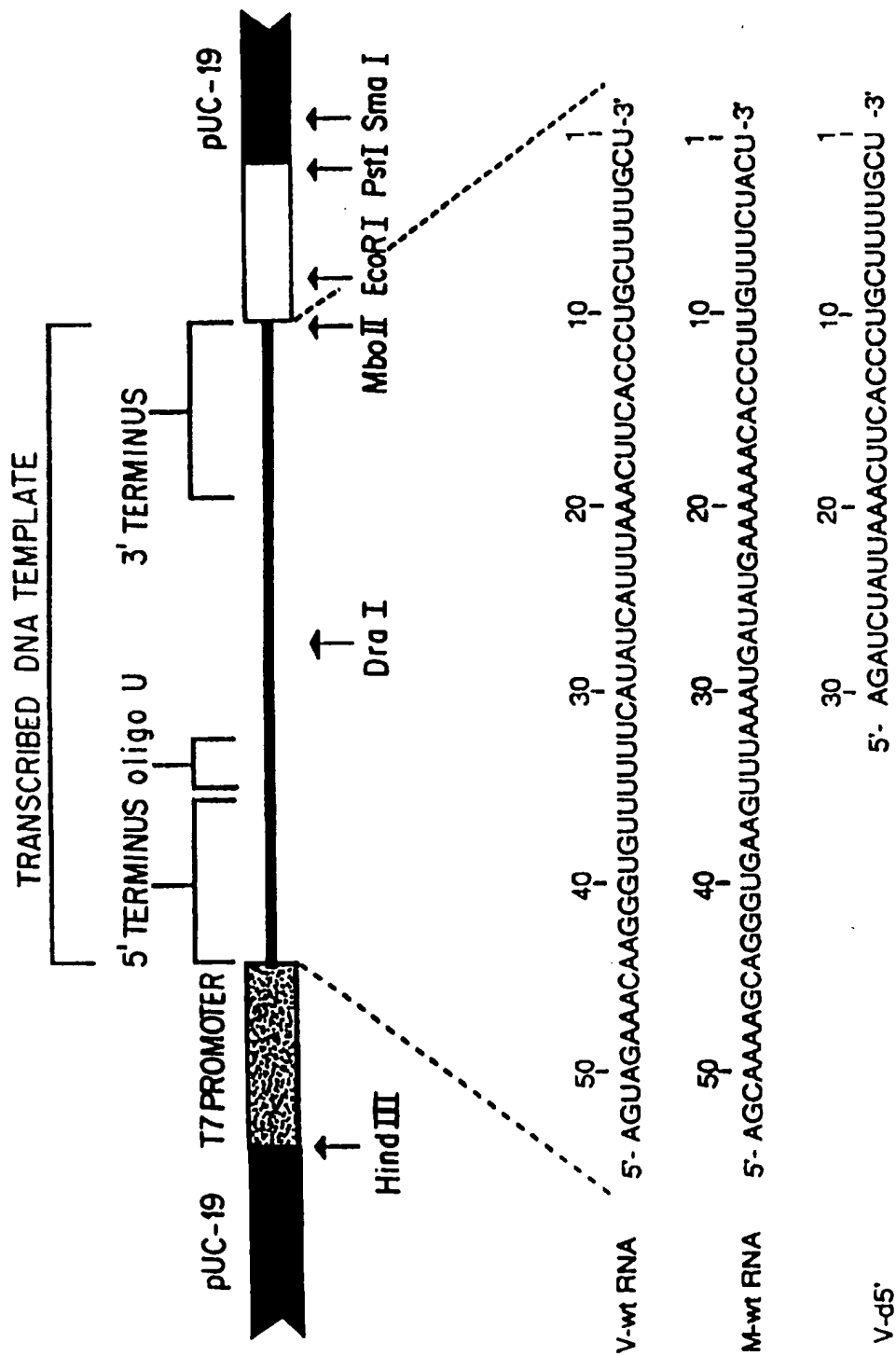


FIG. 2

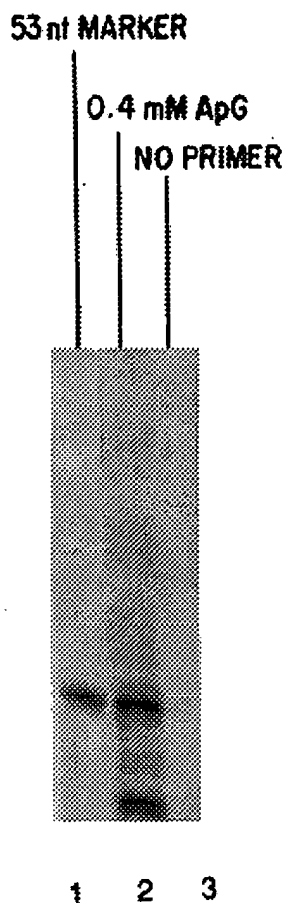


FIG. 3A

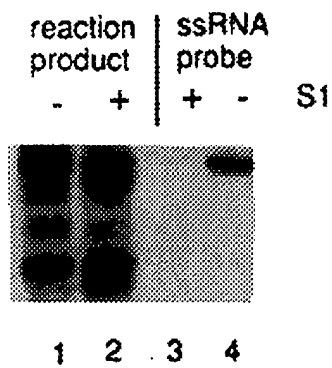


FIG. 3B

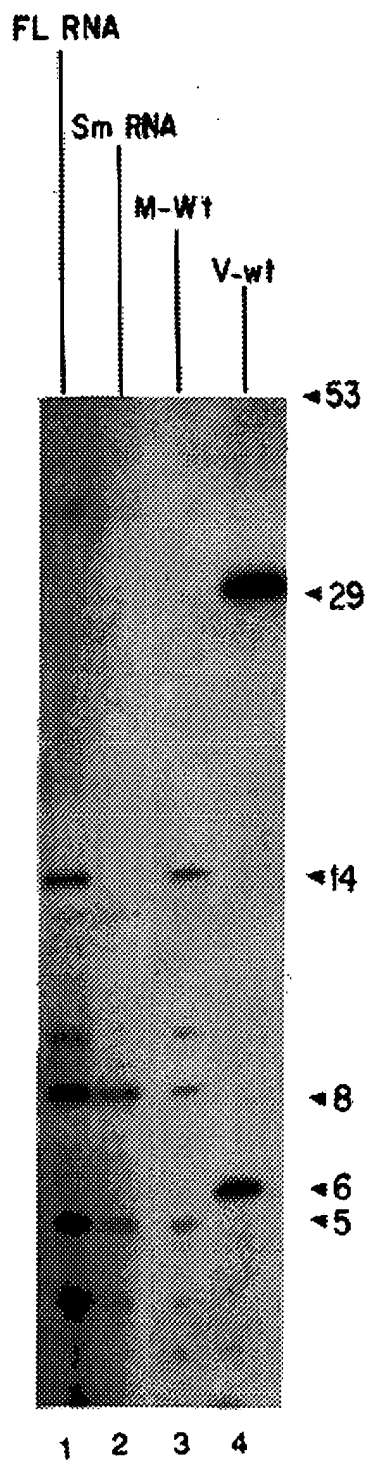


FIG. 3C



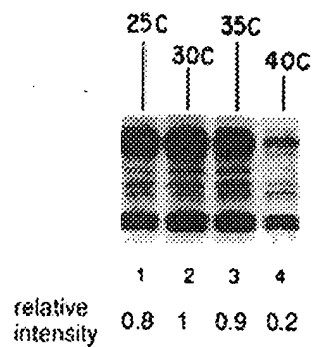


FIG. 4A

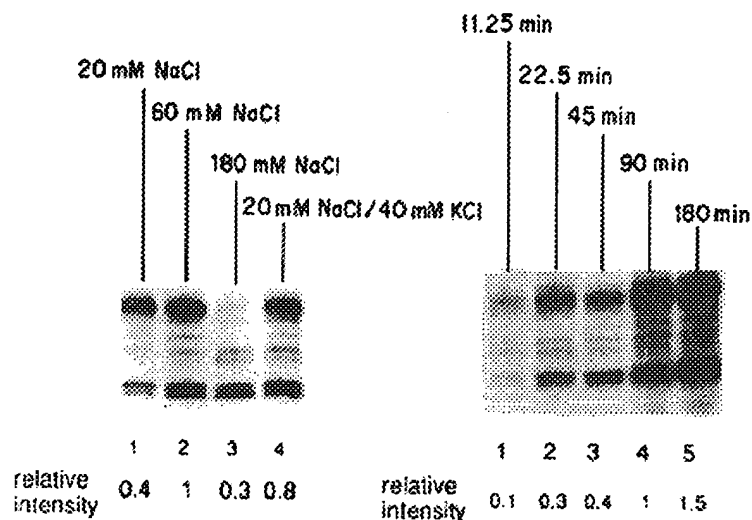


FIG. 4B

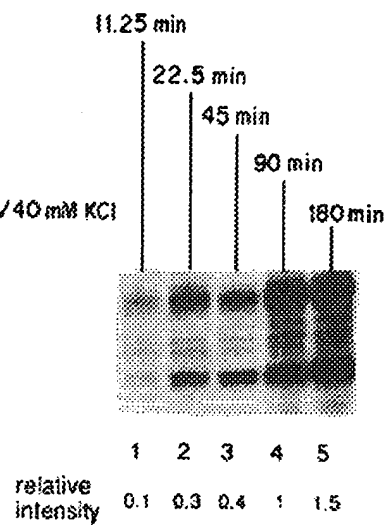
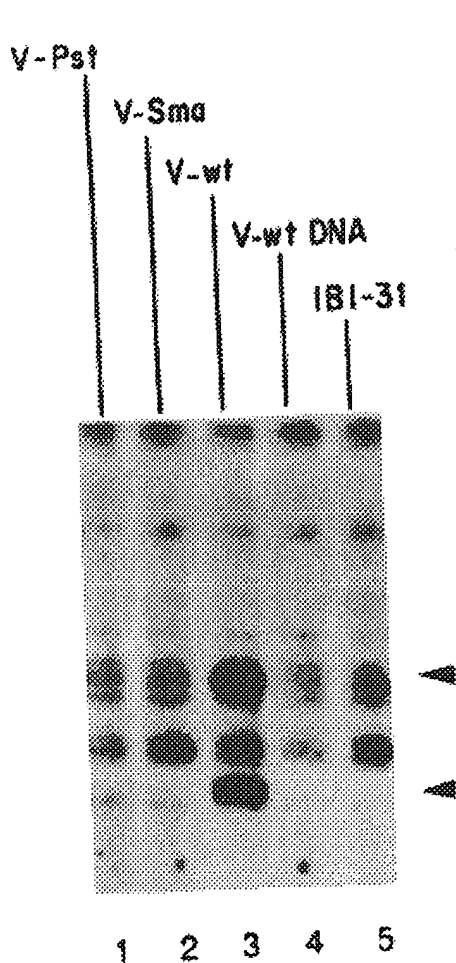
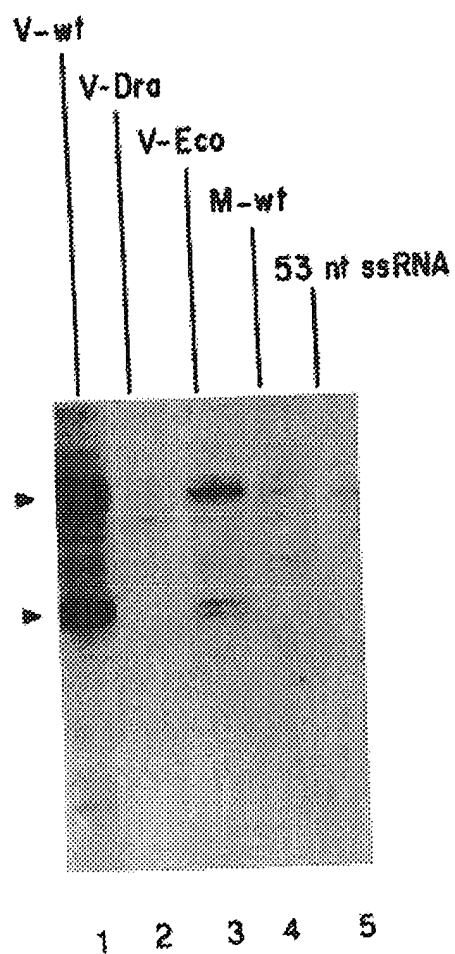
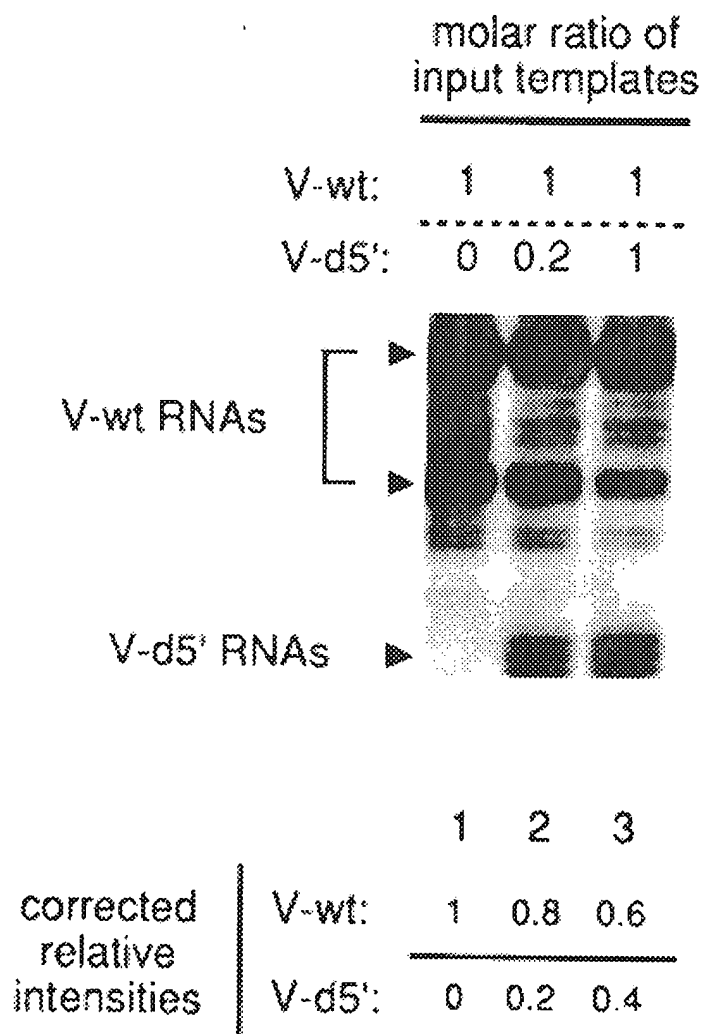
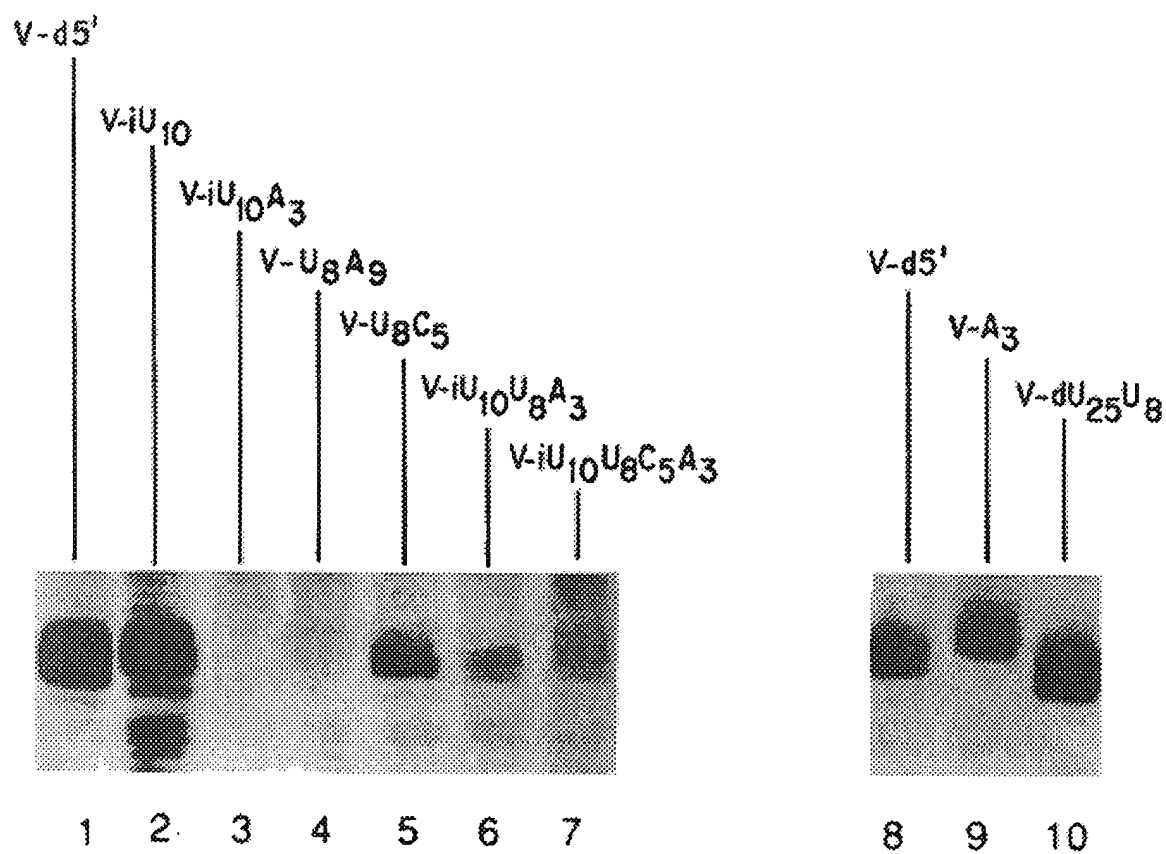


FIG. 4C

**FIG. 5A****FIG. 5B**

**FIG. 6**

**FIG. 7**

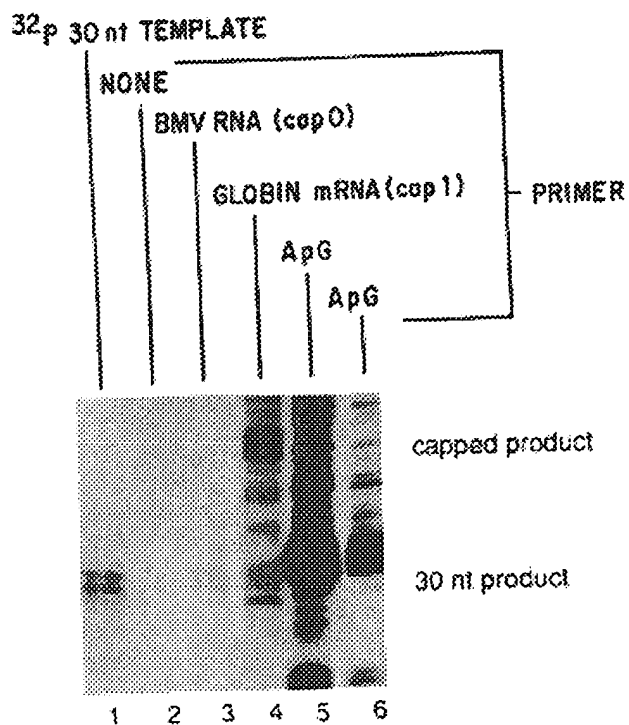


FIG. 8A

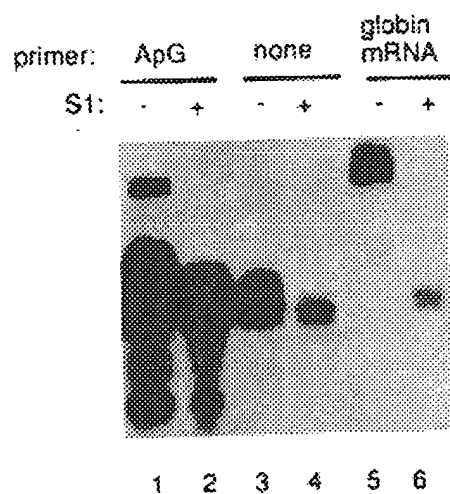


FIG. 8B

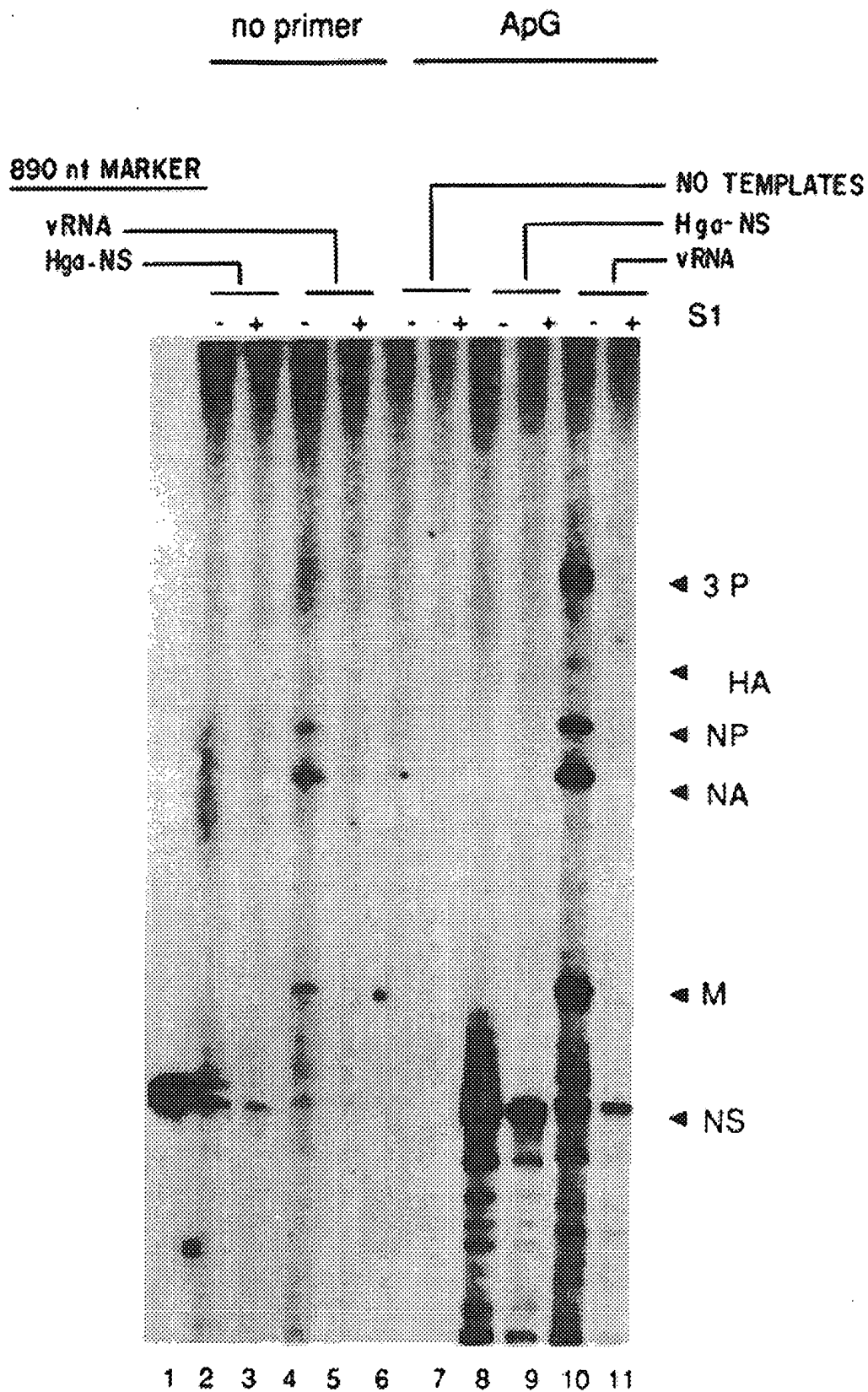
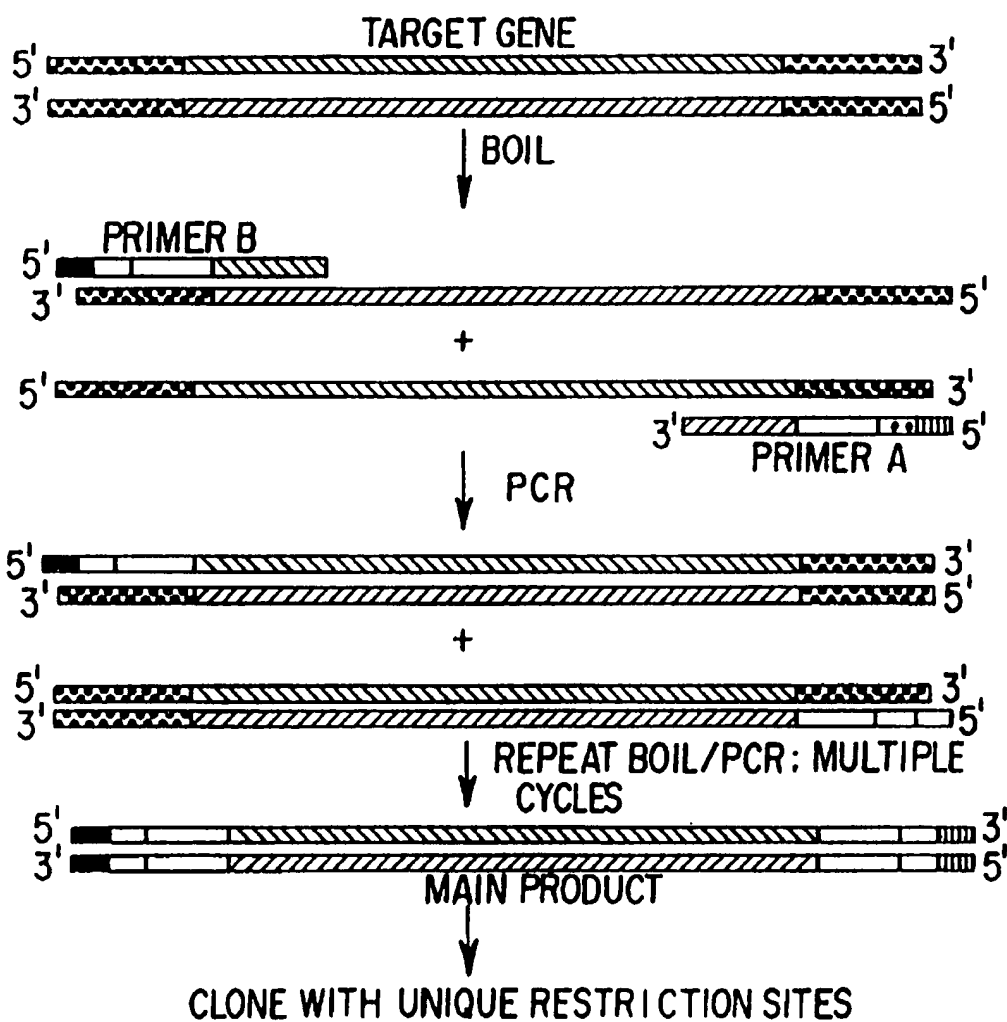


FIG. 9

FIG. 10



FLANKING SEQUENCE	PHAGE PROMOTER
;  UNIQUE RESTRICTION SITES	VIRAL SEQUENCE (5' OR 3')
;  TARGET GENE SEQUENCE	CLASS IIS SITE

FIG. 11A

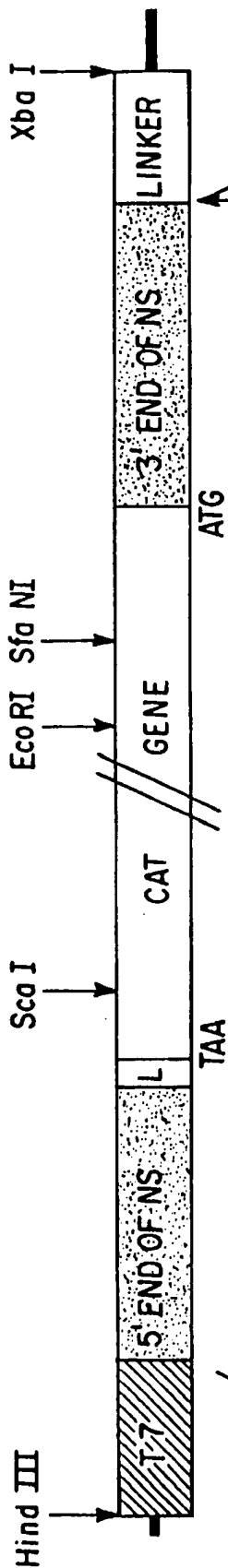
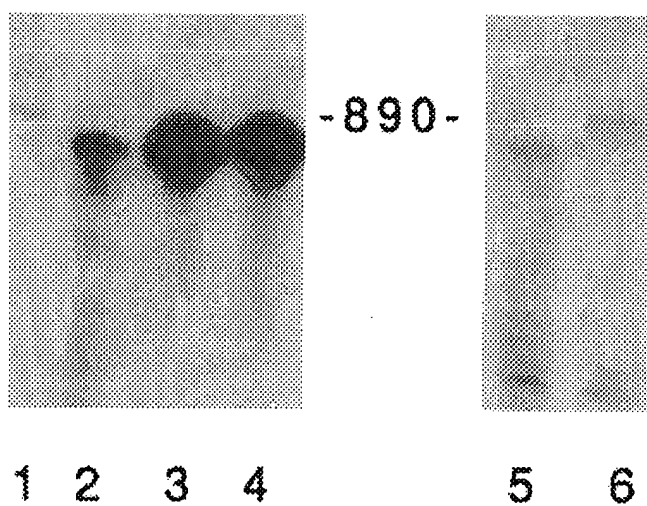


FIG. 11B

5' AGUAGAAACAAGGGUGUUUU CAGAU CUA UUACGCCCGGCC // GUGGUAUACCCAGUGAUUUUUUCUCCAU UAUGUCUUUUGUCACCCUGCUUUGCU 3'



**FIG. 12**



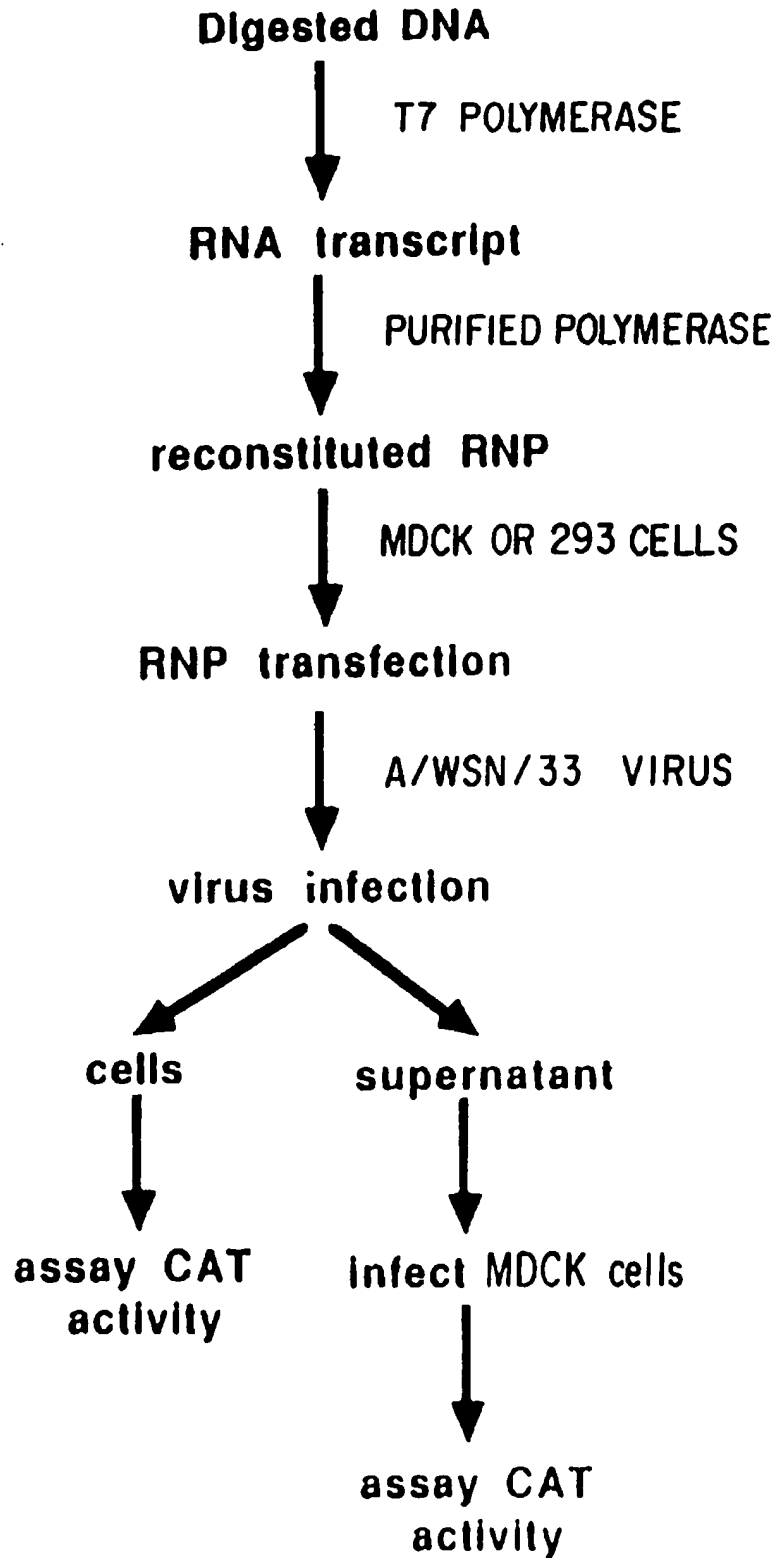


FIG. 13

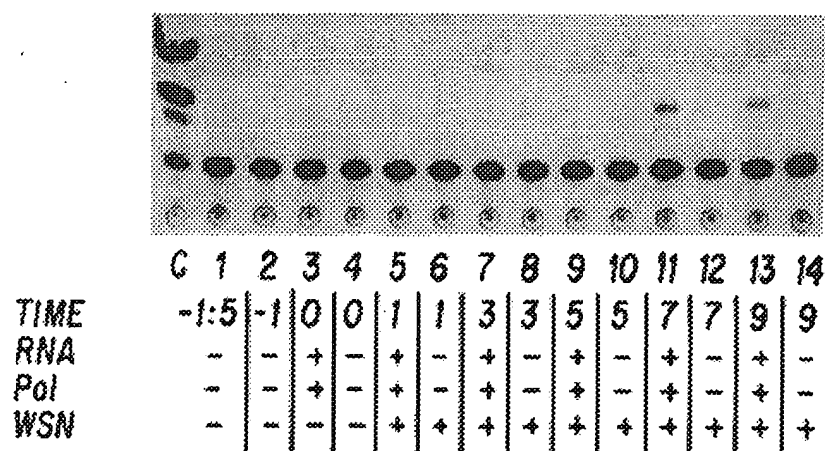


FIG. 14A

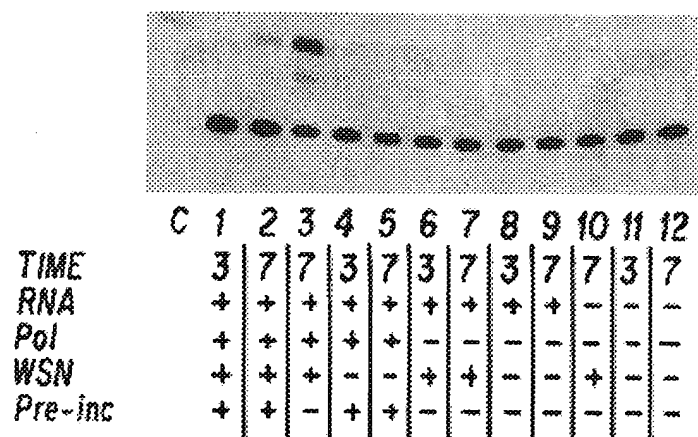
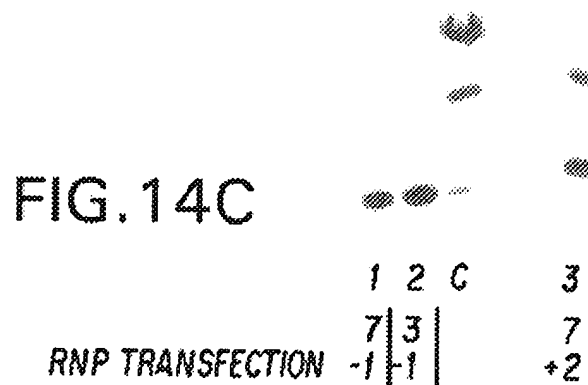
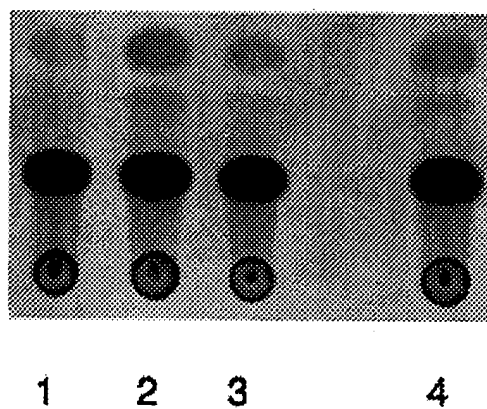


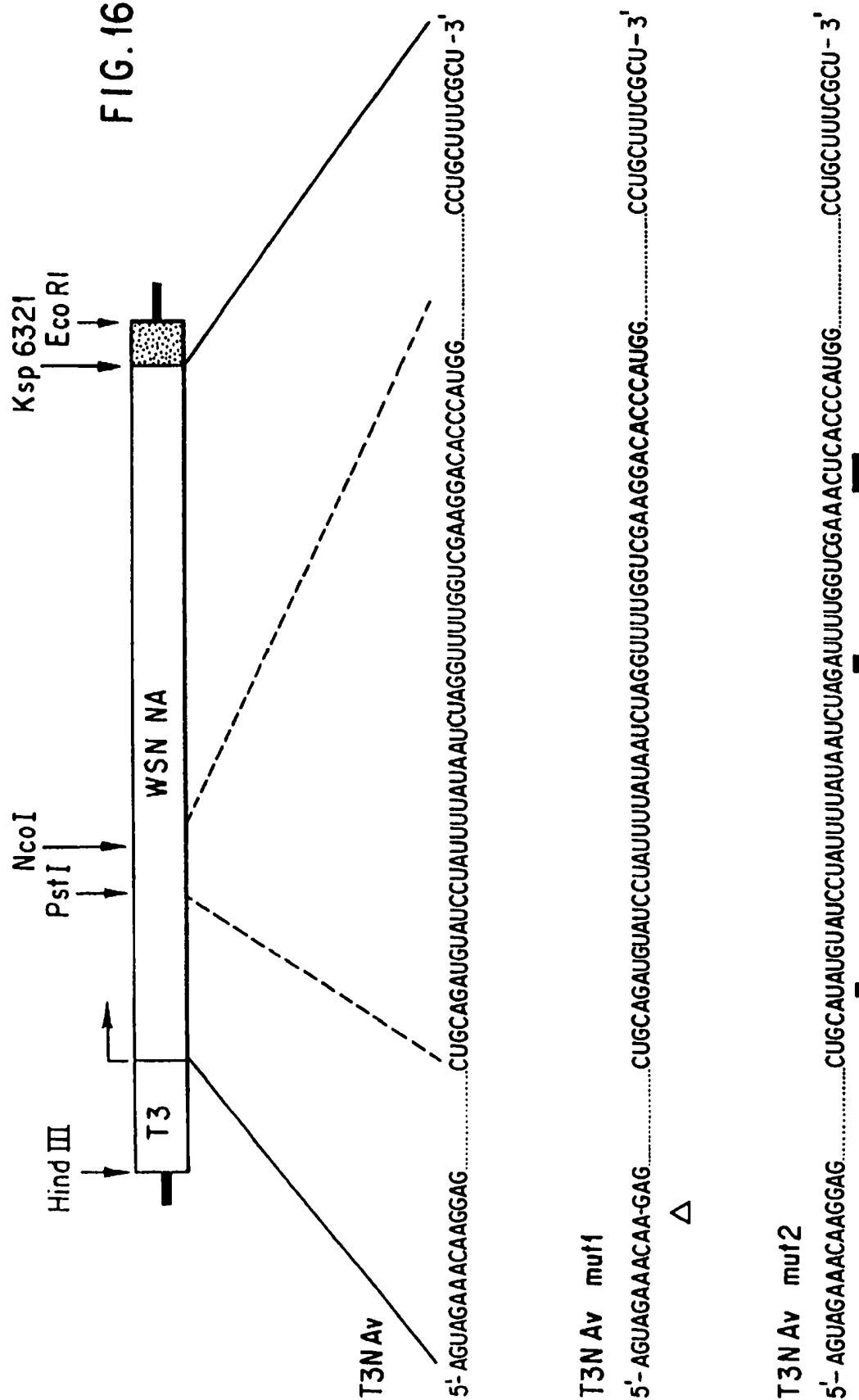
FIG. 14B

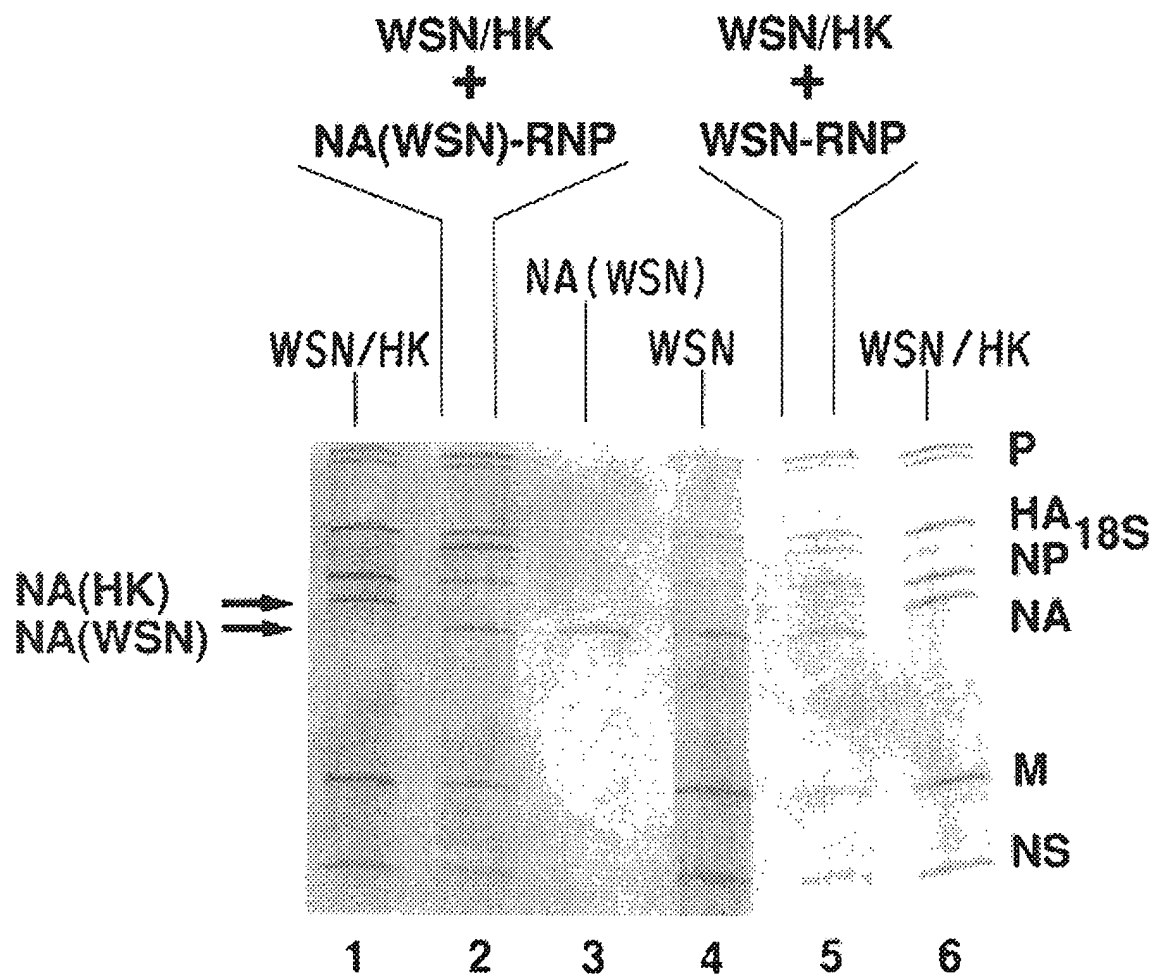


**FIG. 15**



**FIG. 16**



**FIG. 17**

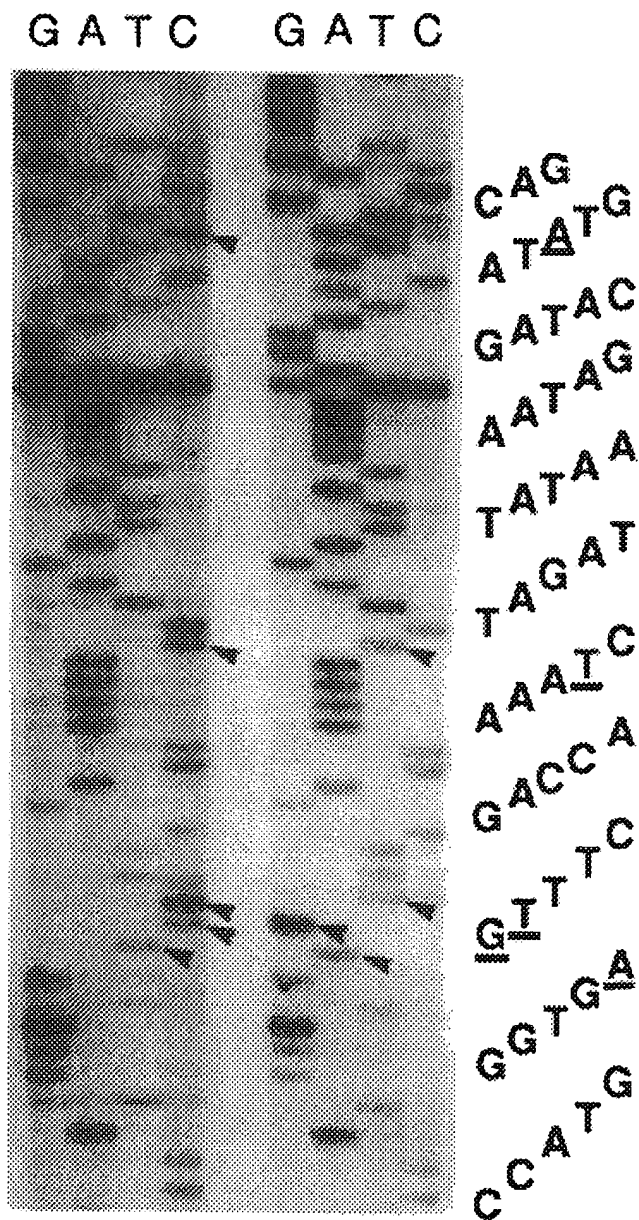
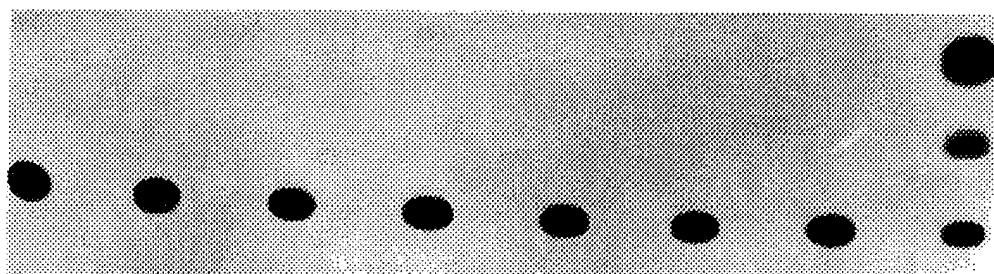
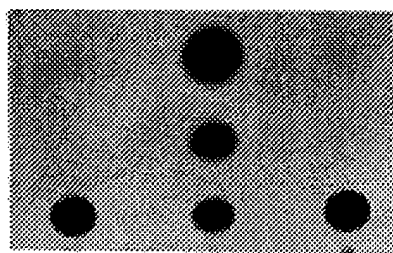
**FIG. 18**

FIG. 19



1	2	3	4	5	6	7	8
C	NAKED RNA	NO VIRUS	INF. VIRUS	PB2	PB2 PB1	PB2 PB1 PA	PB2 PB1 PA NP
	NO VIRUS						



9	10	11
PB2 PA NP	PB2 PB1 PA NP	PB1 PA NP



FIG. 20A

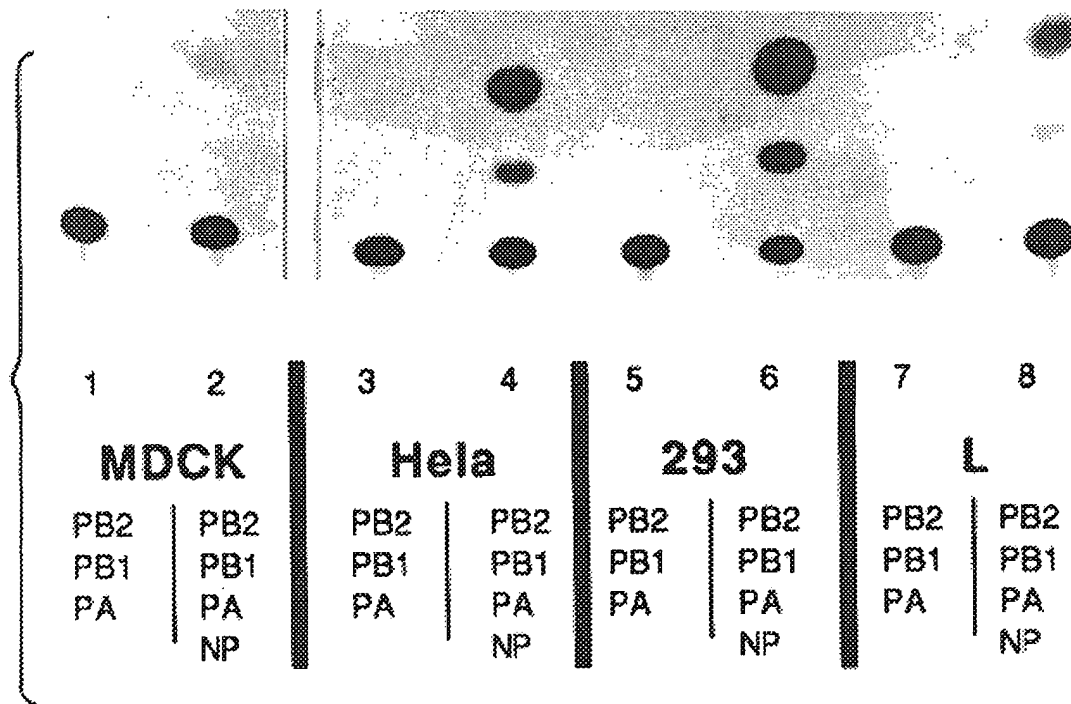


FIG. 20B

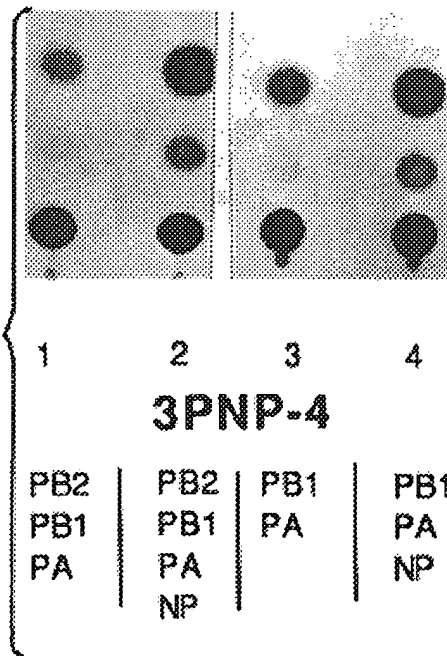
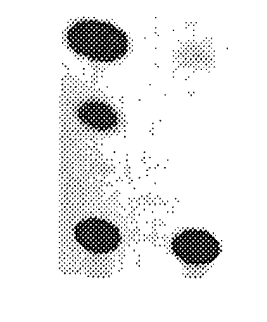


FIG. 20C



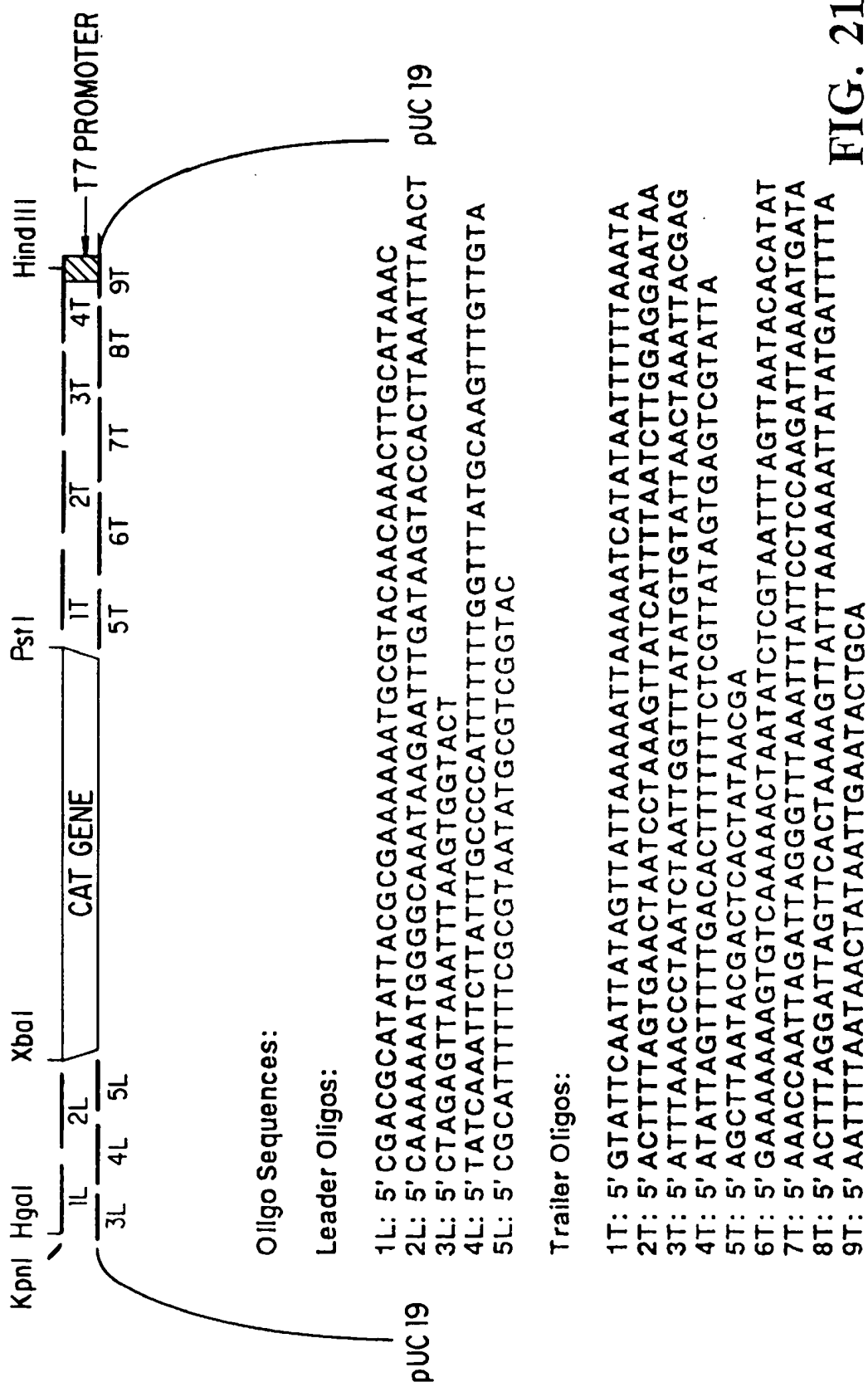


FIG. 21

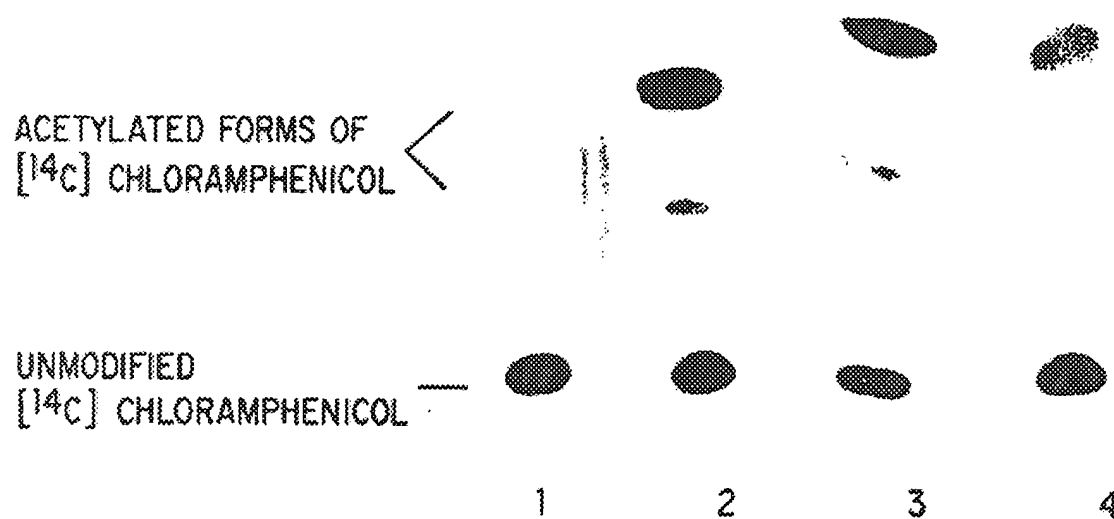
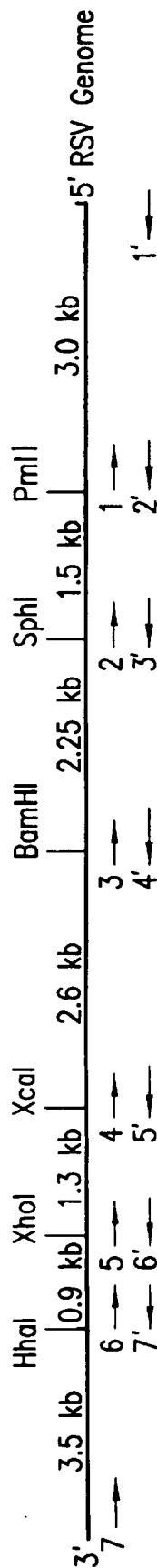


FIG.22



## Primer Sequences:

1: 5' GTTTAACACGTGGTGAG  
 2: 5' ACATATAGGCATGCACC  
 3: 5' GACAAATGGATCCCATTT  
 4: 5' TGGTTGGTATACCAAGTGT  
 5: 5' TACCAAGAGCTCGAGTCA  
 6: 5' TTACCATATGCGCTAATGT  
 7: 5' ACGCGAAAAAATGCGTACA

1': 5' ACGAGAAAAAAAGTGTCAC  
 2': 5' CTCACCCACGTGTTAAAC  
 3': 5' GGTGCATGCCCTATATGT  
 4': 5' AATGGGATCCATTTTGTC  
 5': 5' AACACTGGTATACCAACCA  
 6': 5' TGACTCGAGCTCTTGGTA  
 7': 5' ACATTAGCGCATATGGTAAA

FIG. 23

## RECOMBINANT NEGATIVE STRAND RNA VIRUS EXPRESSION SYSTEMS

This application is a continuation-in-part of application Ser. No. 08/190,698 filed Feb. 1, 1994, now abandoned, which is a continuation of Ser. No. 07/925,061 filed Aug. 4, 1992 (now abandoned) which is a divisional of Ser. No. 07/527,237 (now U.S. Pat. No. 5,166,057), which is a continuation-in-part of Ser. No. 07/440,053 filed Nov. 21, 1989 (now abandoned) and Ser. No. 07/399,728, filed Aug. 28, 1989, (now abandoned) which are incorporated by reference herein in their entirety.

### 1. INTRODUCTION

The present invention relates to recombinant negative strand virus RNA templates which may be used to express heterologous gene products in appropriate host cell systems and/or to construct recombinant viruses that express, package, and/or present the heterologous gene product. The expression products and chimeric viruses may advantageously be used in vaccine formulations.

The invention is demonstrated by way of examples in which recombinant influenza virus RNA templates containing a heterologous gene coding sequences in the negative-polarity were constructed. These recombinant templates, when combined with purified viral RNA-directed RNA polymerase, were infectious, replicated in appropriate host cells, and expressed the heterologous gene product at high levels. In addition, the heterologous gene was expressed and packaged by the resulting recombinant influenza viruses. The invention is further demonstrated by way of examples in which recombinant respiratory syncytial virus RNA templates containing a heterologous gene coding sequence in the negative-polarity were constructed and used to infect and replicate in appropriate host cells.

### 2. BACKGROUND OF THE INVENTION

A number of DNA viruses have been genetically engineered to direct the expression of heterologous proteins in host cell systems (e.g., vaccinia virus, baculovirus, etc.). Recently, similar advances have been made with positive-strand RNA viruses (e.g., poliovirus). The expression products of these constructs, i.e., the heterologous gene product or the chimeric virus which expresses the heterologous gene product, are thought to be potentially useful in vaccine formulations (either subunit or whole virus vaccines). One drawback to the use of viruses such as vaccinia for constructing recombinant or chimeric viruses for use in vaccines is the lack of variation in its major epitopes. This lack of variability in the viral strains places strict limitations on the repeated use of chimeric vaccinia, in that multiple vaccinations will generate host-resistance to the strain so that the inoculated virus cannot infect the host. Inoculation of a resistant individual with chimeric vaccinia will, therefore, not induce immune stimulation.

By contrast, influenza virus, a negative-strand RNA virus, demonstrates a wide variability of its major epitopes. Indeed, thousands of variants of influenza have been identified; each strain evolving by antigenic drift. The negative-strand viruses such as influenza would be attractive candidates for constructing chimeric viruses for use in vaccines because its genetic variability allows for the construction of a vast repertoire of vaccine formulations which will stimulate immunity without risk of developing a tolerance. However, achieving this goal has been precluded by the fact that, to date, it has not been possible to construct recombinant or chimeric negative-strand RNA particles that are infectious.

### 2.1. The Influenza Virus

Virus families containing enveloped single-stranded RNA of the negative-sense genome are classified into groups having non-segmented genomes (Paramyxoviridae, Rhabdoviridae) or those having segmented genomes (Orthomyxoviridae, Bunyaviridae and Arenaviridae). The Orthomyxoviridae family, described in detail below, and used in the examples herein, contains only the viruses of influenza, types A, B and C.

The influenza virions consist of an internal ribonucleoprotein core (a helical nucleocapsid) containing the single-stranded RNA genome, and an outer lipoprotein envelope lined inside by a matrix protein (M). The segmented genome of influenza A consists of eight molecules (seven for influenza C) of linear, negative polarity, single-stranded RNAs which encode ten polypeptides, including: the RNA-directed RNA polymerase proteins (PB2, PB1 and PA) and nucleoprotein (NP) which form the nucleocapsid; the matrix proteins (M1, M2); two surface glycoproteins which project from the lipoprotein envelope: hemagglutinin (HA) and neuraminidase (NA); and nonstructural proteins whose function is unknown (NS 1 and NS2). Transcription and replication of the genome takes place in the nucleus and assembly occurs via budding on the plasma membrane. The viruses can reassort genes during mixed infections.

Influenza virus adsorbs via HA to sialyloligosaccharides in cell membrane glycoproteins and glycolipids. Following endocytosis of the virion, a conformational change in the HA molecule occurs within the cellular endosome which facilitates membrane fusion, thus triggering uncoating. The nucleocapsid migrates to the nucleus where viral mRNA is transcribed as the essential initial event in infection. Viral mRNA is transcribed by a unique mechanism in which viral endonuclease cleaves the capped 5'-terminus from cellular heterologous mRNAs which then serve as primers for transcription of viral RNA templates by the viral transcriptase. Transcripts terminate at sites 15 to 22 bases from the ends of their templates, where oligo(U) sequences act as signals for the template-independent addition of poly(A) tracts. Of the eight viral mRNA molecules so produced, six are monocistronic messages that are translated directly into the proteins representing HA, NA, NP and the viral polymerase proteins, PB2, PB1 and PA. The other two transcripts undergo splicing, each yielding two mRNAs which are translated in different reading frames to produce M1, M2, NS1 and NS2. In other words, the eight viral mRNAs code for ten proteins: eight structural and two nonstructural. A summary of the genes of the influenza virus and their protein products is shown in Table I below.

TABLE I

Influenza Virus Genome RNA Segments and Coding Assignments <sup>a</sup>					
Segment	Length <sup>b</sup> (Nucleotides)	Encoded Poly-peptide <sup>c</sup>	Length <sup>d</sup> (Amino Acids)	Molecules Per Virion	Comments
1	2341	PB2	759	30-60	RNA transcriptase component; host cell RNA cap binding
2	2341	PB1	757	30-60	RNA transcriptase component; initiation of transcription; endonuclease activity?

TABLE I-continued

Influenza Virus Genome RNA Segments and Coding Assignments <sup>a</sup>					
Segment	Length <sup>b</sup> (Nucleotides)	Encoded Poly-peptide <sup>c</sup>	Length <sup>d</sup> (Amino Acids)	Molecules Per Virion	Comments
3	2233	PA	716	30-60	RNA transcriptase component; elongation of mRNA chains?
4	1778	HA	566	500	Hemagglutinin; trimer; envelope glycoprotein; mediates attachment to cells
5	1565	NP	498	100	Nucleoprotein; associated with RNA; structural component of RNA transcriptase
6	1413	NA	454	100	Neuraminidase; tetramer; envelope glycoprotein
7	1027	M <sub>1</sub>	252	3000	Matrix protein; lines inside of envelope
		M <sub>2</sub>	96		Structural protein in plasma membrane; spliced mRNA
		?	79		Unidentified protein
8	890	NS <sub>1</sub>	230		Nonstructural protein; function unknown
		NS <sub>2</sub>	121		Nonstructural protein; function unknown; spliced mRNA

<sup>a</sup>Adapted from R. A. Lamb and P. W. Choppin (1983), Reproduced from the Annual Review of Biochemistry, Volume 52, 467-506.

<sup>b</sup>For A/PR/8/34 strain.

<sup>c</sup>Determined by biochemical and genetic approaches.

<sup>d</sup>Determined by nucleotide sequence analysis and protein sequencing.

Following transcription, virus genome replication is the second essential event in infection by negative-strand RNA viruses. As with other negative-strand RNA viruses, virus genome replication in influenza is mediated by virus-specified proteins. It is hypothesized that most or all of the viral proteins that transcribe influenza virus mRNA segments also carry out their replication. All viral RNA segments have common 3' and 5' termini, presumably to enable the RNA-synthesizing apparatus to recognize each segment with equal efficiency. The mechanism that regulates the alternative uses (i.e., transcription or replication) of the same complement of proteins (PB2, PB1, PA and NP) has not been clearly identified but appears to involve the abundance of free forms of one or more of the nucleocapsid proteins, in particular, the NP. The nucleus appears to be the site of virus RNA replication, just as it is the site for transcription.

The first products of replicative RNA synthesis are complementary copies (i.e., plus-polarity) of all influenza virus genome RNA segments (cRNA). These plus-stranded copies (anti-genomes) differ from the plus-strand mRNA transcripts in the structure of their termini. Unlike the mRNA transcripts, the anti-genomic cRNAs are not capped and methylated at the 5' termini, and are not truncated and polyadenylated at the 3' termini. The cRNAs are coterminal with their negative strand templates and contain all the genetic information in each genomic RNA segment in the complementary form. The cRNAs serve as templates for the synthesis of genomic negative-strand vRNAs.

The influenza virus negative strand genomes (vRNAs) and antigenomes (cRNAs) are always encapsidated by

nucleocapsid proteins; the only unencapsidated RNA species are virus mRNAs. In contrast to the other enveloped RNA viruses, nucleocapsid assembly appears to take place in the nucleus rather than in the cytoplasm. The virus matures by budding from the apical surface of the cell incorporating the M protein on the cytoplasmic side or inner surface of the budding envelope. The HA and NA become glycosylated and incorporated into the lipid envelope. In permissive cells, HA is eventually cleaved, but the two resulting chains remain united by disulfide bonds.

It is not known by what mechanism one copy of each of the eight genomic viral RNAs is selected for incorporation into each new virion. Defective interfering (DI) particles are often produced, especially following infection at high multiplicity.

## 2.2. RNA Directed RNA Polymerase

The RNA-directed RNA polymerases of animal viruses have been extensively studied with regard to many aspects of protein structure and reaction conditions. However, the elements of the template RNA which promote optimal expression by the polymerase could only be studied by inference using existing viral RNA sequences. This promoter analysis is of interest since it is unknown how a viral polymerase recognizes specific viral RNAs from among the many host-encoded RNAs found in an infected cell.

Animal viruses containing plus-sense genome RNA can be replicated when plasmid-derived RNA is introduced into cells by transfection (for example, Racaniello et al. 1981, Science 214: 916-919; Levis, et al., 1986, Cell 44: 137-145). In the case of poliovirus, the purified polymerase will replicate a genome RNA in *in vitro* reactions and when this preparation is transfected into cells it is infectious (Kaplan, et al., 1985, Proc. Natl. Acad. Sci. USA 82: 8424-8428). However, the template elements which serve as transcription promoter for the poliovirus-encoded polymerase are unknown since even RNA homopolymers can be copied (Ward, et al., 1988, J. Virol. 62: 558-562). SP6 transcripts have also been used to produce model defective interfering (DI) RNAs for the Sindbis viral genome. When the RNA is introduced into infected cells, it is replicated and packaged. The RNA sequences which were responsible for both recognition by the Sindbis viral polymerase and packaging of the genome into virus particles were shown to be within 162 nucleotides (nt) of the 5' terminus and 19 nt of the 3' terminus of the genome (Levis, et al., 1986, Cell 44: 137-145). In the case of brome mosaic virus (BMV), a positive strand RNA plant virus, SP6 transcripts have been used to identify the promoter as a 134 nt tRNA-like 3' terminus (Dreher, and Hall, 1988, J. Mol. Biol. 201: 31-40). Polymerase recognition and synthesis were shown to be dependent on both sequence and secondary structural features (Dreher, et al., 1984, Nature 311: 171-175).

The negative-sense RNA viruses have been refractory to study of the sequence requirements of the replicase. The purified polymerase of vesicular stomatitis virus is only active in transcription when virus-derived ribonucleoprotein complexes (RNPs) are included as template (De and Banerjee, 1985, Biochem. Biophys. Res. Commun. 126: 40-49); Emerson and Yu, 1975, J. Virol. 15: 1348-1356; Naito, and Ishihama, 1976, J. Biol. Chem. 251: 4307-4314). RNPs have been reconstituted from naked RNA of VSV DI particles using infected cell extracts as protein source. These RNPs were then replicated when added back to infected cells (Mirakhur, and Peluso, 1988, Proc. Natl. Acad. Sci. USA 85: 7511-7515). With regard to influenza viruses, it

was recently reported that naked RNA purified from virus was used to reconstitute RNPs. The viral nucleocapsid and polymerase proteins were gel-purified and renatured on the viral RNA using thioredoxin (Szewczyk, et al., 1988, Proc. Natl. Acad. Sci. USA, 85: 7907-7911). However, these authors did not show that the activity of the preparation was specific for influenza viral RNA, nor did they analyze the signals which promote transcription.

During the course of influenza virus infection the polymerase catalyzes three distinct transcription activities. These include the synthesis of (a) subgenomic mRNA, which contains a 5' cap and a 3' poly-A tail; (b) a full length plus-strand or anti-genome (cRNA) copied from the genome RNA; and (c) genomic vRNA synthesized from the full length cRNA (reviewed in Ishihama and Nagata, 1988, CRC Crit. Rev. Biochem. 23: 27-76; and Krug, Transcription and replication of influenza viruses. In: Genetics of influenza viruses, Ed., Palese, P. and Kingsbury, D. W. New York, Springer-Verlag, 1983, p. 70-98). Viral proteins PB2, PB1 and PA are thought to catalyze all influenza virus-specific RNA synthesis when in the presence of excess nucleocapsid protein (NP; see above reviews). These polymerase functions have been studied using RNP cores derived from detergent-disrupted virus, and RNPs from the nuclear extracts of infected cells. Transcription from the RNPs derived from disrupted virus occurs when primed with either dinucleotide adenylyl-(3'-5')-guanosine (ApG) or capped mRNAs. The plus sense mRNA products have terminated synthesis 17-20 nucleotides upstream of the 5' terminus of the RNA template and have been processed by the addition of poly A tails. These products cannot serve as template for the viral-sense genome since they lack terminal sequences (Hay, et al., 1977, Virology 83: 337-355). RNPs derived from nuclear extracts of infected cells also synthesize polyadenylated mRNA in the presence of capped RNA primers. However, if ApG is used under these conditions, both RNAs, polyadenylated and full length cRNA, can be obtained (Beaton and Krug, 1986, Proc. Natl. Acad. Sci. USA 83: 6282-6286; Takeuchi, et al., 1987, J. Biochem. 101: 837-845). Recently it was shown that replicative synthesis of cRNA could occur in the absence of exogenous primer if the nuclear extract was harvested at certain times post infection. In these same preparations the synthesis of negative-sense vRNA from a cRNA template was also observed (Shapiro and Krug, 1988, J. Virol. 62: 2285-2290). The synthesis of full length cRNA was shown to be dependent upon the presence of nucleocapsid protein (NP) which was free in solution (Beaton and Krug, 1986, Proc. Natl. Acad. Sci. USA 83: 6282-6286; Shapiro and Krug, 1988, J. Virol. 62: 2285-2290). These findings led to the suggestion that the regulatory control between mRNA and cRNA synthesis by the RNP complex is based on the requirement for there being an excess of soluble NP (Beaton and Krug, 1986, Proc. Natl. Acad. Sci. USA 83: 6282-6286).

Another line of investigation has focused on the preparation of polymerase-RNA complexes derived from RNPs from detergent-disrupted virus. When the RNP complex is centrifuged through a CsCl-glycerol gradient, the RNA can be found associated with the three polymerase (P) proteins at the bottom of the gradient. Near the top of the gradient, free NP protein can be found (Honda, et al., 1988, J. Biochem. 104: 1021-1026; Kato, et al., 1985, Virus Research 3, 115-127). The purified polymerase-RNA complex (bottom of gradient), is active in initiating ApG-primed synthesis of RNA, but fails to elongate to more than 12-19 nucleotides. When fractions from the top of the gradient containing the NP protein are added back to the polymerase-

RNA complex, elongation can ensue (Honda, et al., 1987, J. Biochem. 102: 41-49). These data suggest that the NP protein is needed for elongation, but that initiation can occur in the absence of NP.

It has been shown that the genomic RNA of influenza viruses is in a circular conformation via base-pairing of the termini to form a panhandle of 15 to 16 nt (Honda, et al., 1988, J. Biochem. 104: 1021-1026; Hsu, et al., 1987, Proc. Natl. Acad. Sci. USA 84: 8140-8144). Since the viral polymerase was found bound to the panhandle, this led to the suggestion that a panhandle structure was required for recognition by the viral polymerase (Honda, et al., 1988, J. Biochem. 104: 1021-1026). Therefore, it was hypothesized in these two reports that the promoter for the viral RNA polymerase was the double stranded RNA in panhandle conformation.

### 3. SUMMARY OF THE INVENTION

Recombinant negative-strand viral RNA templates are described which may be used with purified RNA-directed RNA polymerase complex to express heterologous gene products in appropriate host cells and/or to rescue the heterologous gene in virus particles. Both influenza and respiratory syncytial viral templates are exemplified. The RNA templates are prepared by transcription of appropriate DNA sequences with a DNA-directed RNA polymerase. The resulting RNA templates are of the negative-polarity and contain appropriate terminal sequences which enable the viral RNA-synthesizing apparatus to recognize the template.

As demonstrated by the examples described herein, recombinant negative-sense influenza RNA templates may be mixed with purified viral polymerase proteins and nucleoprotein (i.e., the purified viral polymerase complex) to form infectious recombinant RNPs. These can be used to express heterologous gene products in host cells or to rescue the heterologous gene in virus particles by cotransfection of host cells with recombinant RNPs and virus. Alternatively, the recombinant RNA templates or recombinant RNPs may be used to transfect transformed cell lines that express the RNA dependent RNA-polymerase and allow for complementation. Additionally, a non-virus dependent replication system for influenza virus is also described. Vaccinia vectors expressing influenza virus polypeptides were used as the source of proteins which were able to replicate and transcribe synthetically derived RNPs. The minimum subset of influenza virus protein needed for specific replication and expression of the viral RNP was found to be the three polymerase proteins (PB2, PB1 and PA) and the nucleoprotein (NP). This suggests that the nonstructural proteins, NS1 and NS2, are not absolutely required for the replication and express of viral RNP.

The expression products and/or chimeric virions obtained may advantageously be utilized in vaccine formulations. The use of recombinant influenza for this purpose is especially attractive since influenza demonstrates tremendous strain variability allowing for the construction of a vast repertoire of vaccine formulations. The ability to select from thousands of influenza variants for constructing chimeric viruses obviates the problem of host resistance encountered when using other viruses such as vaccinia. In addition, since influenza stimulates a vigorous secretory and cytotoxic T cell response, the presentation of foreign epitopes in the influenza virus background may also provide for the induction of secretory immunity and cell-mediated immunity.

## 3.1. Definitions

As used herein, the following terms will have the meanings indicated:

cRNA=anti-genomic RNA  
 HA=hemagglutinin (envelope glycoprotein)  
 M=matrix protein (lines inside of envelope)  
 MDCK=Madin Darby canine kidney cells  
 MDBK=Madin Darby bovine kidney cells  
 moi=multiplicity of infection  
 NA=neuraminidase (envelope glycoprotein)  
 NP=nucleoprotein (associated with RNA and required for polymerase activity)  
 NS=nonstructural protein (function unknown)  
 nt=nucleotide  
 PA, PB1, PB2 =RNA-directed RNA polymerase components  
 RNP=ribonucleoprotein (RNA, PB2, PB1, PA and NP)  
 rRNP=recombinant RNP  
 vRNA=genomic virus RNA  
 viral polymerase complex=PA, PB1, PB2 and NP  
 WSN=influenza A/WSN/33 virus  
 WSN-HK virus: reassortment virus containing seven genes from WSN virus and the NA gene from influenza A/HK/8/68 virus

## 4. DESCRIPTION OF THE FIGURES

FIG. 1. Purification of the polymerase preparation. RNP cores were purified from whole virus and then subjected to CsCl-glycerol gradient centrifugation. The polymerase was purified from fractions with 1.5 to 2.0M CsCl. Samples were then analyzed by polyacrylamide gel electrophoresis on a 7–14% linear gradient gel in the presence of 0.1% sodium dodecylsulfate followed by staining with silver. Protein samples contained 1.4  $\mu$ g whole virus (lane 1), 0.3  $\mu$ g whole virus (lane 2), 5  $\mu$ l of RNP cores (lane 3) and 25  $\mu$ l RNA polymerase (lane 4). Known assignments of the proteins are indicated at the left.

FIG. 2. Plasmid constructs used to prepare RNA templates. The plasmid design is depicted with the solid box representing pUC-19 sequences, the hatched box represents the truncated promoter specifically recognized by bacteriophage T7 RNA polymerase, the solid line represents the DNA which is transcribed from plasmids which have been digested with MboII. The white box represents sequences encoding the recognition sites for MboII, EcoRI and PstI, in that order. Sites of cleavage by restriction endonucleases are indicated. Beneath the diagram, the entire sequences of RNAs which result from synthesis by T7 RNA polymerase from MboII-digested plasmid are given. The V-wt RNA has the identical 5' and 3' termini as found in RNA segment 8 of influenza A viruses, separated by 16 "spacer" nucleotides. The RNA, M-wt, represents the exact opposite strand, or "message-sense", of V-wt. Restriction endonuclease sites for DraI, EcoRI, PstI and SmaI are indicated. T7 transcripts of plasmids cleaved by these enzymes result in, respectively, 32, 58, 66 and 91 nucleotide long RNAs. The sequences of V-d5' RNA are indicated. The plasmid design is essentially the same as that used for the V-wt RNA except for the minor changes in the "spacer" sequence. The point mutants of V-d5' RNAs which were studied are indicated in Table I.

FIG. 3. Analysis of products of influenza viral polymerase. FIG. 3A: Polymerase reaction mixtures containing 0.4 mM ApG (lane 2) or no primer (lane 3) were electro-

phoresed on 8% polyacrylamide gels containing 7.7M urea. FIG. 3B: The nascent RNA is resistant to single-stranded specific nuclease S1. Following the standard polymerase reaction, the solutions were diluted in nuclease S1 buffer (lane 1) and enzyme was added (lane 2). As control for S1 digestion conditions, radioactively labeled single-stranded V-wt RNA was treated with nuclease S1 (lane 3) or with buffer alone (lane 4). FIG. 3C: Ribonuclease T1 analysis of gel-purified reaction products. The reaction products of the viral polymerase using the V-wt RNA template was subjected to electrophoresis on an 8% polyacrylamide gel. The 53 nt band and the smaller transcript were excised and eluted from the gel matrix. These RNAs were digested with RNase T1 and analyzed by electrophoresis on a 20% polyacrylamide gel containing 7.7M urea. For comparison, T7 transcripts of M-wt and V-wt RNAs which had been synthesized in the presence of  $\alpha$ - $^{32}$ P-UTP were also analyzed with RNase T1. The predicted radiolabeled oligonucleotides of the control RNAs are indicated. Lane 1, 53 nucleotide full length (FL) product; lane 2, 40–45 nucleotide smaller (Sm) RNA product; lane 3, M-wt RNA labeled by incorporation of  $^{32}$ P-MP; and lane 4, V-wt RNA labeled as in lane 3.

FIG. 4. Optimal reaction conditions for the viral polymerase. FIG. 4A: Reactions with V-wt template were assembled on ice and then incubated at the indicated temperatures for 90 minutes. FIG. 4B: Reactions with the V-wt template were prepared in parallel with the indicated NaCl or KCl concentrations and were incubated at 30° C. for 90 minutes. FIG. 4C: A single reaction with the V-wt template was incubated at 30° C., and at the indicated times, samples were removed and immediately processed by phenol-chloroform extraction. All gels contained 8% polyacrylamide with 7.7M urea.

FIG. 5. Template specificity of the viral polymerase. FIG. 5A: The viral polymerase reaction requires 3' terminal promoter sequences. Different template RNAs were used in reactions under standard conditions. Lane 1, the V-Pst RNA, which is identical to V-wt except it has a 13 nt extension at the 3' end; lane 2, V-Sma RNA, which has a 38 nt extension at the 3' end; lane 3, V-wt RNA; lane 4, a DNA polynucleotide with identical sequence as the V-wt RNA; lane 5, and 80 nt RNA generated by bacteriophage T3 RNA polymerase transcription of a pIBI-31 plasmid digested with HindIII. The autoradiograph was overexposed in order to emphasize the absence of specific reaction products when these other templates were used. FIG. 5B: 10 ng of each template RNA were incubated with the viral polymerase and the products were then subjected to electrophoresis on 8% polyacrylamide gels containing 7.7M urea. Lane 1, V-wt RNA; lane 2, V-Dra RNA; lane 3, V-Eco RNA; lane 4, M-wt RNA are shown; and lane 5, a 53nt marker oligonucleotide.

For the exact sequence differences refer to FIG. 2 and Section 6.1 et seq.

FIG. 6. The RNA promoter does not require a terminal panhandle. Polymerase reaction using two template RNAs. Each reaction contained 5 ng of V-wt RNA. As a second template the reactions contained 0 ng (lane 1), 0.6 ng (lane 2), and 3.0 ng (lane 3) of V-d5' RNA. The resulting molar ratios are as indicated in the figure. The reaction products were analyzed on an 8% polyacrylamide gel in the presence of 7.7M urea. Following densitometry analysis of autoradiographs, the relative intensity of each peak was corrected for the amount of radioactive UMP which is incorporated in each product.

FIG. 7. Specificity of promoter sequences. RNAs which lacked the 5' terminus and contained point mutations (Table



II) were compared with V-d5' RNA in standard polymerase reactions. The right panel is from a separate reaction set. Quantitative comparisons is outlined in Table II.

FIG. 8. High concentration polymerase preparations are active in cap-endonuclease primed and in primeness RNA synthesis reactions. FIG. 8A: Primer specificity of the high concentration enzyme. Radioactively synthesized 30 nt template is in lane 1. Reactions using 20 ng of V-d5' RNA and 5  $\mu$ l of viral polymerase contained as primer: no primer (lane 2); 100 ng BMV RNA (De and Banerjee, 1985, Biochem. Biophys. Res. Commun. 6: 40-49) containing a cap 0 structure (lane 3); 100 ng rabbit globin mRNA, containing a cap 1 structure, (lane 4); and 0.4 mM ApG (lane 5). A lighter exposure of lane 5 is shown as lane 6. FIG. 8B: Nuclease S1 analysis of gel-purified RNAs. Products from reactions using as primer ApG (lanes 1 and 2); no primer (lanes 3 and 4); or globin mRNA (lanes 5 and 6) were electrophoresed in the absence of urea and the appropriate gel piece was excised and the RNA was eluted. This RNA was then digested with nuclease S1 (lanes 2, 4, and 6) and the products were denatured and analyzed on an 8% polyacrylamide gel containing 7.7M urea.

FIG. 9. Genomic length RNA synthesis from reconstituted RNPs. Reaction products using 10  $\mu$ l of polymerase and as template 890 nt RNA identical to the sequence of segment 8 of virus A/WSN/33 and RNA extracted from A/PR/8/34 virus were analyzed on a 4% polyacrylamide gel containing 7.7M urea. In lane 1, the 890 nt template synthesized radioactively by T7 RNA polymerase is shown. The 890 nt plasmid-derived RNA was used as template in lanes 2, 3, 8 and 9. RNA extracted from virus was used as template in lanes 4, 5, 10 and 11. No template was used in lanes 6 and 7. No primer was used in lanes 2 to 5, and ApG was used as primer in lanes 6 to 11. Reaction products were treated with nuclease S1 in lanes 3, 5, 7, 9 and 11.

FIG. 10. Diagrammatic representation of a PCR-directed mutagenesis method which can be used to replace viral coding sequences within viral gene segments.

FIG. 11. (A). Diagrammatic representation of relevant portions of pIVCAT1. The various domains are labeled and are, from left to right; a truncated T7 promoter; the 5' nontranslated end of influenza A/PR/8/34 virus segment 8 (22 nucleotides); 8 nucleotides of linker sequence; the entire CAT gene coding region (660 nucleotides) the entire 3' nontranslated end of influenza A/PR/8/34 virus segment 8 (26 nucleotides); and linker sequence containing the HgaI restriction enzyme site. Relevant restriction enzyme sites and start and stop sites for the CAT gene are indicated. (B) The 716 base RNA product obtained following HgaI digestion and transcription of pIVCAT1 by T7 RNA polymerase. Influenza viral sequences are indicated by bold letters, CAT gene sequences by plain letters, and linker sequences by italics. The triplets—in antisense orientation—representing the initiation and termination codons of the CAT gene are indicated by arrow and underline, respectively.

FIG. 12. RNA products of T7 polymerase transcription and in vitro influenza virus polymerase transcription. Lanes 1-4: polyacrylamide gel analysis of radiolabeled T7 polymerase transcripts from pIVCAT1, and pHgaNS. Lanes 5 and 6: Polyacrylamide gel analysis of the radiolabeled products of in vitro transcription by purified influenza A polymerase protein using unlabeled IVACAT1 RNA and HgaNS RNA templates. Lane 1: HgaNS RNA of 80 nt. Lanes 2-4: different preparations of IVACAT1 RNA. Lane 5: viral polymerase transcript of IVACAT1 RNA. Lane 6: viral polymerase transcript of HgaNS RNA.

FIG. 13. Schematic of the RNP-transfection and passaging experiments.

FIG. 14. CAT assays of cells RNP-transfected with IVA-CAT1 RNA. (A) Time course of RNP-transfection in 293 cells. Cells were transfected at -1 hour with the recombinant RNP and infected with virus at 0 hour. Cells were harvested at the indicated time points and assayed for CAT activity. (B) Requirements for RNP-transfection of 293 cells. Parameters of the reaction mixtures were as indicated. (C) RNP-transfection of MDCK cells. MDCK cells were transfected with IVACAT1 RNA-polymerase at either -1 hour or +2 hours relative to virus infection. Cells were harvested and CAT activity assayed at the indicated times. Components/conditions of the reaction were as indicated. "Time" indicates the time point of harvesting the cells. T=0 marks the time of addition of helper virus. "RNA" represents the IVACAT1 RNA. "Pol" is the purified influenza A/PR/8/34 polymerase protein complex. "WSN" indicates the influenza A/WSN/33 helper virus. "Pre-Inc." indicates preincubation of RNA and polymerase in transcription buffer at 30° C. for 30 min. "RNP transfection" indicates the time of RNP transfection relative to virus infection. "+/-" indicate presence or absence of the particular component/feature. "C" indicates control assays using commercially available CAT enzyme (Boehringer-Mannheim).

FIG. 15. CAT activity in MDCK cells infected with recombinant virus. Supernatant from RNP-transfected and helper virus-infected MDCK cells was used to infect fresh MDCK cells. The inoculum was removed 1 hour after infection, cells were harvested 11 hours later and CAT activity was assayed. Lane 1: extract of cells infected with helper virus only. Lane 2: extract of cells infected with 100  $\mu$ l of supernatant from RNP-transfected and helper virus-infected MDCK cells. Lane 3: Supernatant (80  $\mu$ l) of cells from lane 2. Lane 4: Same as lane 2 except that helper virus (MOI 4) was added to inoculum. In contrast to experiments shown in FIG. 4, the assays contained 20  $\mu$ l of <sup>14</sup>C chloramphenicol.

FIG. 16. Diagram of relevant portions of the neuraminidase (NA) gene contained in plasmids used for transfection experiments. The pUC19 derived plasmid pT3NAv contains the influenza A/WSN/33 virus NA gene and a truncated promoter specifically recognized by bacteriophage T3 RNA polymerase. The T3 promoter used is truncated such that the initial transcribed nucleotide (an adenine) corresponds to the 5' adenine of the WSN NA gene. At the 3' end of the cDNA copy of the NA gene, a Ksp632I restriction enzyme site was inserted such that the cleavage site occurs directly after the 3' end of the NA gene sequence. A 1409 nucleotide long transcript was obtained following Ksp632I digestion and transcription by T3 RNA polymerase of pT3NAv (as described in Section 8.1, infra). The 15 5' terminal nucleotides, the 52 nucleotides corresponding to the region between the restriction endonuclease sites NcoI and PstI and the 12 3' terminal nucleotides are shown. The transcript of pT3NAv mut 1 is identical to that of pT3NAv except for a single deletion, eleven nucleotides downstream from the 5' end of the wild type RNA. The transcript of the pT3NAv mut 2 is identical to that of pT3NAv except for 5 mutations located in the central region (indicated by underline). These five mutations do not change the amino acid sequence in the open reading frame of the gene. The serine codon UCC at position 887-889 (plus sense RNA) was replaced with the serine codon AGU in the same frame. The numbering of nucleotides follows Hiti et al., 1982, J. Virol., 41: 730-734.

FIG. 17. Polyacrylamide gel electrophoresis of RNAs purified from rescued influenza viruses. RNA transcripts of

pT3NAs (FIG. 16) of phenol-extracted RNA derived from influenza A/WSN/33 virus was mixed with purified polymerase preparations following the protocol described in Section 6.1.1, *infra*. These reconstituted RNPs were then transfected into MDBK cells which had been infected one hour earlier with WSN-HK helper virus. The medium, containing 28  $\mu$ g/ml plasminogen, was harvested after 16 hours and virus was amplified and plaqued on MDBK cells in the absence of protease. Virus obtained from plaques was then further amplified in MDBK cells and RNA was phenol-extracted from purified virus preparations as described in Sections 6.1 et seq. and 7.1 et seq. RNAs were separated on 2.8% polyacrylamide-0.075 % bisacrylamide gels containing 7.7M urea in TBE buffer and visualized by silverstaining as described in Section 6.1 et seq. Lanes 1 and 6: WSN-HK virus RNA. Lane 2: RNA of virus which was rescued from MDBK cells following RNP-transfection with pT3NAv derived NA RNA and infection with helper virus WSN-HK. Lane 3: NA RNA transcribed in vitro from pT3NAv. Lane 4: RNA of control WSN virus. Lane 5: RNA of virus which was rescued from MDBK cells following RNP-transfection with phenol-extracted WSN virus RNA and infection with helper virus WSN-HK.

FIG. 18. Sequence analysis of RNA obtained from rescued influenza virus containing five site-specific mutations. Following infection with the WSN-HK helper virus, MDBK cells were RNP-transfected with T3NAv mut 2 RNA which was obtained by transcription from pT3NAv mut 2. Following overnight incubation in the presence of 28  $\mu$ g/ml plasminogen, medium was used for propagation and plaquing on MDBK cells in the absence of protease. Virus from plaques was then amplified and RNA was obtained following phenol-extraction of purified virus. Rescue of the mutant NA gene into virus particles was verified through direct RNA sequencing using 5'-TACGAGGAAATGTTCTGTTA-3' as primer (corresponding to position 800-819; Hiti et al., J. Virol., 41: 730-734) and reverse transcriptase (Yamashita et al., 1988, Virol. 163: 112-122). Sequences shown correspond to position 878-930 in the NA gene (Hiti et al., J. Virol. 41: 730-734). The arrows and the underlined nucleotides indicate the changes in the mutant RNA compared to the wild type RNA. Left: Control RNA obtained from influenza A/WSN/33 virus. Right: RNA of mutant virus rescued from MDBK cells which were RNP-transfected with T3NAv mut 2 RNA and infected with helper virus WSN-HK.

FIG. 19. CAT expression in vaccinia virus-infected/IVACAT-1 RNP transfected cells. Approximately  $10^6$  mouse C127 cells in 35 mm dishes were infected with mixtures of recombinant vaccinia viruses (Smith et al., 1986) at an M.O.I. of approximately 10 for each vector. After 1.5 hours, synthetic IVACAT-1 RNP was transfected into the virus-infected cells as described (Lutjyes et al., 1989). Cells were incubated overnight, harvested and assayed for CAT activity according to standard procedures (Gorman et al., 1982). The assays contained 0.05  $\mu$ Ci [ $^{14}$ C] chloramphenicol, 20  $\mu$ l of 40 mM acetyl-CoA (Boehringer and 50  $\mu$ l of cell extracts in 0.25 M Tris buffer (pH 7.5). Incubation times were approximately 4 hours. The labels under the lane numbers indicate the treatment of cells. Lanes 1-control; 2-naked RNA transfection (no polymerase added), no helper virus infection; 3-RNP transfection, no helper virus; 4-RNP transfection, influenza virus as helper; Lanes 5-11-RNP transfection, vaccinia virus vectors as helper viruses express the indicated influenza virus proteins.

FIG. 20. Test of various cell lines. A) Cells were infected with vaccinia vectors expressing the PB2, PB1 and PA

proteins (Lanes 1,3,5,7) or the PB2, PB1, PA and NP proteins (Lanes 2,4,6,8), transfected with IVACAT-1 RNP and examined for CAT activity as described. Lanes 1,2: Maden-Darby Canine Kidney (MDCK) cell; 3,4: HeLa cells, 5,6: 293 cells (Graham et al., 1977 J. Gen. Virol., 36: 59-72); 7,8 L cells. B) Cell line 3 PNP-4 was used as host cell. Shown under each lane is the influenza viral proteins expressed in each sample. C) 293 cells were infected with the four required vaccinia and transfected with synthetic RNP made using IVA-CAT-1 (lane 1) or IVA-CAT-2 (lane 2) RNA. After overnight incubation, cells were harvested and CAT assays were performed.

FIG. 21. Schematic representation of the RSV/CAT construct (pRSVA2CAT) used in rescue experiments. The approximate 100 nt long leader and 200 nt long trailer regions of RSV were constructed by the controlled annealing of synthetic oligonucleotides containing partial overlapping complementarity. The overlapping leader oligonucleotides are indicated by the 1L-5L shown in the construct. The overlapping trailer nucleotides are indicated by the 1T-9T shown in the construct. The nucleotide sequences of the leader and trailer oligonucleotides are shown below the construct. The fully formed leader and trailer DNAs were ligated into purified CAT gene DNA at the indicated XbaI and PstI sites respectively. This entire construct was then ligated into KpnI/HindIII digested pUC19. The inclusion of a T7 promoter sequence and a HgaI site flanking the trailer and leader sequences respectively allowed in vitro synthesis of RSV/CAT RNA transcripts containing the precise genomic sequence 3' and 5' ends.

FIG. 22. Thin layer chromatogram (TLC) showing the CAT activity present in 293 cell extracts following infection and transfection with RNA transcribed from the RSV/CAT construct shown in FIG. 1. Confluent monolayers of 293 cells in 6-well plates ( $\sim 10^6$  cells) were infected with either RSV A2 or B9320 at an m.o.i. of 0.1-1.0 pfu cell $^{-1}$ . At 1 hour post infection the cells were transfected with 5-10  $\mu$ g of CAT/RSV using the Transfectace<sup>TM</sup> protocol of Gibco BRL. At 24 hours post infection the infected/transfected monolayers were harvested and processed for subsequent CAT assay according to Current Protocols in Molecular Biology, Vol. 1, Chapter 9.6.2; Gorman, et al., (1982) Mol. Cell. Biol. 2:1044-1051.

Lanes 1, 2, 3, and 4 show the CAT activity present in (1) uninfected 293 cells, transfected with CAT/RSV RNA, (2) RSV-A2 infected 293 cells, transfected with CAT/RSV RNA, (3) RSV-B9320 infected 293 cells transfected with CAT/RSV RNA, (4) RSV-A2 infected 293 cells, co-infected with supernatant from (2) above. The CAT activity observed in each lane was produced from  $\frac{1}{2}$  of the total cellular extract from  $10^6$  cells.

FIG. 23. Schematic representation of the RSV strain A2 genome showing the relative positions of the primer pairs used for the synthesis of cDNAs comprising the entire genome. The endonuclease sites used to splice these clones together are indicated; these sites were present in the native RSV sequence and were included in the primers used for cDNA synthesis. Approximately 100 ng of viral genomic RNA was used in RT/PCR reactions for the separate synthesis of each of the seven cDNAs. The primers for the first and second strand cDNA synthesis from the genomic RNA template are also shown. For each cDNA, the primers for the first strand synthesis are nos. 1-7 and the primers for the second strand synthesis are nos. 1'-7'.

## 5. DESCRIPTION OF THE INVENTION

This invention relates to the construction and use of recombinant negative strand viral RNA templates which

may be used with viral RNA-directed RNA polymerase to express heterologous gene products in appropriate host cells and/or to rescue the heterologous gene in virus particles. The RNA templates may be prepared by transcription of appropriate DNA sequences using a DNA-directed RNA polymerase such as bacteriophage T7, T3 or the Sp6 polymerase. Using influenza, for example, the DNA is constructed to encode the message- sense of the heterologous gene sequence flanked upstream of the ATG by the complement of the viral polymerase binding site/promoter of influenza, i.e., the complement of the 3'-terminus of a genome segment of influenza. For rescue in virus particles, it may be preferred to flank the heterologous coding sequence with the complement of both the 3'-terminus and the 5'-terminus of a genome segment of influenza. After transcription with a DNA-directed RNA polymerase, the resulting RNA template will encode the negative polarity of the heterologous gene sequence and will contain the vRNA terminal sequences that enable the viral RNA-directed RNA polymerase to recognize the template.

The recombinant negative sense RNA templates may be mixed with purified viral polymerase complex comprising viral RNA-directed RNA polymerase proteins (the P proteins) and nucleoprotein (NP) which may be isolated from RNP cores prepared from whole virus to form "recombinant RNPs" (rRNPs). These rRNPs are infectious and may be used to express the heterologous gene product in appropriate host cells or to rescue the heterologous gene in virus particles by cotransfection of host cells with the rRNPs and virus. Alternatively, the recombinant RNA templates may be used to transfect transformed cell lines that express the RNA-directed RNA polymerase proteins allowing for complementation.

The invention is demonstrated by way of working examples in which RNA transcripts of cloned DNA containing the coding region—in negative sense orientation—of the chloramphenicol acetyltransferase (CAT) gene flanked by the 22 5' terminal and the 26 3' terminal nucleotides of the influenza A/PR/8/34 virus NS RNA were mixed with isolated influenza A virus polymerase proteins. This reconstituted ribonucleoprotein (RNP) complex was transfected into MDCK (or 293) cells, which were infected with influenza virus. CAT activity was negligible before and soon after virus infection, but was demonstrable by seven hours post virus infection. When cell supernatant containing budded virus from this "rescue" experiment was used to infect a new monolayer of MDCK cells, CAT activity was also detected, suggesting that the RNA containing the recombinant CAT gene had been packaged into virus particles. These results demonstrate the successful use of recombinant negative strand viral RNA templates and purified RNA-dependent RNA polymerase to reconstitute recombinant influenza virus RNP. Furthermore, the data suggest that the 22 5' terminal and the 26 3' terminal sequences of the influenza A virus RNA are sufficient to provide the signals for RNA transcription, A replication and for packaging of RNA into influenza virus particles.

Using this methodology we also demonstrated the rescue of synthetic RNAs, derived from appropriate recombinant plasmid DNAs, into stable and infectious influenza viruses. In particular, RNA corresponding to the neuraminidase (NA) gene of influenza A/WSN/33 virus (WSN) was transcribed in vitro from plasmid DNA and, following the addition of purified influenza virus polymerase complex, was transfected into MDBK cells. Superinfection with helper virus lacking the WSN NA gene resulted in the release of virus containing the WSN NA gene. We then introduced five point

mutations into the WSN NA gene by cassette mutagenesis of the plasmid DNA. Sequence analysis of the rescued virus revealed that the genome contained all five mutations present in the mutated plasmid. This technology can be used to create viruses with site-specific mutations so that influenza viruses with defined biological properties may be engineered.

The ability to reconstitute RNPs in vitro allows the design of novel chimeric influenza viruses which express foreign genes. One way to achieve this goal involves modifying existing influenza virus genes. For example, the HA gene may be modified to contain foreign sequences in its external domains. Where the heterologous sequence are epitopes or antigens of pathogens, these chimeric viruses may be used to induce a protective immune response against the disease agent from which these determinants are derived. In addition to modifying genes coding for surface proteins, genes coding for nonsurface proteins may be altered. The latter genes have been shown to be associated with most of the important cellular immune responses in the influenza virus system (Townsend et al., 1985, Cell 42: 475-482). Thus, the inclusion of a foreign determinant in the NP or the NS gene of an influenza virus may—following infection—induce an effective cellular immune response against this determinant. Such an approach may be particularly helpful in situations in which protective immunity heavily depends on the induction of cellular immune responses (e.g., malaria, etc.).

Another approach which would permit the expression of foreign proteins (or domains of such proteins) via chimeric influenza viruses concerns the introduction of complete heterologous genes into the virus. Influenza virus preparations with more than eight RNA segments have previously been described (Nayak, D. et al. in *Genetics of Influenza Virus*, P. Palese and D. W. Kingsbury, eds., Springer-Verlag, Vienna, pp. 255-279). Thus, chimeric influenza viruses with nine or more RNA segments may be viable, and correct packaging of such chimeric viruses may readily occur.

The invention may be divided into the following stages solely for the purpose of description and not by way of limitation: (a) construction of recombinant RNA templates; b) expression of heterologous gene products using the recombinant RNA templates; and (c) rescue of the heterologous gene in recombinant virus particles. For clarity of discussion, the invention is described in the subsections below using influenza. Any strain of influenza (e.g., A, B, C) may be utilized. However, the principles may be analogously applied to construct other negative strand RNA virus templates and chimeric viruses including, but not limited to paramyxoviruses, such as parainfluenza viruses, measles viruses, respiratory syncytial virus; bunyaviruses; arena viruses; etc. Section 9 describes and exemplifies the application of these principles to the construction of respiratory syncytial virus templates and chimeric respiratory syncytial viruses. A particularly interesting virus system that can be used in accordance with the invention are the orthomyxo-like insect virus called Dhoir (Fuler, 1987, *Virology* 160: 81-87).

### 5.1. Construction of the Recombinant RNA Templates

Heterologous gene coding sequences flanked by the complement of the viral polymerase binding site/promoter, e.g., the complement of 3'-influenza virus terminus, or the complements of both the 3'- and 5'-influenza virus termini may be constructed using techniques known in the art. Recombinant DNA molecules containing these hybrid

sequences can be cloned and transcribed by a DNA-directed RNA polymerase, such as bacteriophage T7, T3 or the Sp6 polymerase and the like, to produce the recombinant RNA templates which possess the appropriate viral sequences that allow for viral polymerase recognition and activity.

One approach for constructing these hybrid molecules is to insert the heterologous coding sequence into a DNA complement of an influenza virus genomic segment so that the heterologous sequence is flanked by the viral sequences required for viral polymerase activity; i.e., the viral polymerase binding site/promoter, hereinafter referred to as the viral polymerase binding site. In an alternative approach, oligonucleotides encoding the viral polymerase binding site, e.g., the complement of the 3'-terminus or both termini of the virus genomic segments can be ligated to the heterologous coding sequence to construct the hybrid molecule. The placement of a foreign gene or segment of a foreign gene within a target sequence was formerly dictated by the presence of appropriate restriction enzyme sites within the target sequence. However, recent advances in molecular biology have lessened this problem greatly. Restriction enzyme sites can readily be placed anywhere within a target sequence through the use of site-directed mutagenesis (e.g., see, for example, the techniques described by Kunkel, 1985, *Proc. Natl. Acad. Sci. U.S.A.* 82:488). Variations in polymerase chain reaction (PCR) technology, described *infra*, also allow for the specific insertion of sequences (i.e., restriction enzyme sites) and allow for the facile construction of hybrid molecules. Alternatively, PCR reactions could be used to prepare recombinant templates without the need of cloning. For example, PCR reactions could be used to prepare double-stranded DNA molecules containing a DNA-directed RNA polymerase promoter (e.g., bacteriophage T3, T7 or Sp6) and the hybrid sequence containing the heterologous gene and the influenza viral polymerase binding site. RNA templates could then be transcribed directly from this recombinant DNA. In yet another embodiment, the recombinant RNA templates may be prepared by ligating RNAs specifying the negative polarity of the heterologous gene and the viral polymerase binding site using an RNA ligase. Sequence requirements for viral polymerase activity and constructs which may be used in accordance with the invention are described in the subsections below.

#### 5.1.1. The Viral 3'-Terminus is Required for Polymerase Activity

The experiments described in Section 6 et seq., *infra*, are the first to define promoter sequences for a polymerase of a negative-sense RNA virus, and it was found that the specificity lies in the 3' terminal 15 nucleotides. These viral polymerase binding site sequences, as well as functionally equivalent sequences may be used in accordance with the invention. For example, functionally equivalent sequences containing substitutions, insertions, deletions, additions or inversions which exhibit similar activity may be utilized. The RNA synthesis by the viral polymerase described *infra* is a model for specific recognition and elongation by the influenza viral polymerase for the following reasons: (a) the polymerase has high activity when primed with ApG, a feature unique to influenza viral polymerase; (b) it has optimal activity at temperature and ionic conditions previously shown to be effective for the viral RNPs; (c) the polymerase is specific for influenza viral sequences on the model RNA templates; (d) the polymerase is active in the cap-endonuclease primed RNA synthesis which is the hallmark of the influenza viral polymerase; (e) recognition of cap donor RNA is specific to cap 1 structures; and (f) genomic RNA segments are specifically copied.

#### 5.1.2. Terminal Panhandle is not Required for Optimal Recognition and Synthesis by the Viral Polymerase

We had previously shown that the influenza viral segment RNAs base-pair at their termini to form panhandle structures. This was achieved by two methods. A cross-linking reagent derivative of psoralen covalently bound the termini of each segment in intact virus or in RNPs from infected cells (Hsu et al., 1987, *Proc. Natl. Acad. Sci. USA* 84: 8140-8144). The treated RNA was seen by electron microscopy to be circular, by virtue of the cross-linked termini. Similarly, the RNA termini in RNPs were found to be sensitive to ribonuclease V1, which recognizes and cleaves double-stranded RNA, and the viral polymerase was found to be bound to both termini in the panhandle conformation (Honda, et al., 1988, *J. Biochem.* 104: 1021-1026). In these studies the panhandle structure of the genomic RNA was shown to exist, and it was inferred to play a role in polymerase recognition. Although the template RNAs used in the examples described, were originally prepared to reveal panhandle-specific protein binding, it was found that the terminal panhandle had no obvious role in the polymerase reactions studied herein.

#### 5.1.3. The RNA Polymerase Preparation Specifically Copies Negative Sense Templates

The viral polymerase was shown to synthesize RNA with optimal efficiency if the template had the "wild-type" negative sense 3' terminus. It was shown that RNAs of unrelated sequence were not copied, and that those with extra polylinker sequences on the 3' end were much less efficiently copied. A DNA of the correct sequence was similarly unsuitable as a template. The reaction was highly specific since the M-wt template was replicated only at very low levels. Even though our source of polymerase was intact virus, this finding was very surprising since it had never been suggested that the polymerase which recognizes the viral sense RNA would not efficiently copy the plus sense strand. Studies are underway to examine the specificity of the polymerase purified from infected cells at times post infection when the complementary RNA is copied into genomic templates. The present data support a model whereby the viral polymerase which copies vRNA is functionally different from that which synthesizes vRNA from cRNA by virtue of their promoter recognition. It is possible that by regulated modification of the polymerase in infected cells it then becomes capable of recognizing the 3' terminus of plus sense RNA. By analyzing promoter mutants we investigated the fine specificity of the reaction and found that the only single mutation which generated a significantly lower level of synthesis was that of V-A<sub>3</sub> RNA. Furthermore, combinations of two or more point changes in positions 3, 5, 8 and 10 greatly lowered synthesis levels.

#### 5.1.4. Insertion of the Heterologous Gene Sequence into the PB2 PB1 PA or NP Gene Segments

The gene segments coding for the PB2, PB1, PA and NP proteins contain a single open reading frame with 24-45 untranslated nucleotides at their 5'-end, and 22-57 untranslated nucleotides at their 3'-end. Insertion of a foreign gene sequence into any of these segments could be accomplished by either a complete replacement of the viral coding region with the foreign gene or by a partial replacement. Complete replacement would probably best be accomplished through the use of PCR-directed mutagenesis. The principle of this mutagenesis method is illustrated in FIG. 10. Briefly, PCR-primer A would contain, from 5' to 3', a unique restriction enzyme site, such as a class IIS restriction enzyme site (i.e., a "shifter" enzyme; that recognizes a specific sequence but

cleaves the DNA either upstream or downstream of that sequence); the entire 3' untranslated region of the influenza gene segment; and a stretch of nucleotides complementary to the carboxy-terminus coding portion of the foreign gene product. PCR-primer B would contain from the 5' to 3' end: a unique restriction enzyme site; a truncated but active phage polymerase sequence; the complement of the entire 5' untranslated region of the influenza gene segment (with respect to the negative sense vRNA); and a stretch of nucleotides corresponding to the 5' coding portion of the foreign gene. After a PCR reaction using these primers with a cloned copy of the foreign gene, the product may be excised and cloned using the unique restriction sites. Digestion with the class IIS enzyme and transcription with the purified phage polymerase would generate an RNA molecule containing the exact untranslated ends of the influenza viral gene segment with a foreign gene insertion. Such a construction is described for the chloramphenicol acetyltransferase (CAT) gene used in the examples described in Section 7 *infra*. In an alternate embodiment, PCR-primed reactions could be used to prepare double-stranded DNA containing the bacteriophage promoter sequence, and the hybrid gene sequence so that RNA templates can be transcribed directly without cloning.

Depending on the integrity of the foreign gene product and the purpose of the construction, it may be desirable to construct hybrid sequences that will direct the expression of fusion proteins. For example, the four influenza virus proteins, PB2, PB1, PA or NP are polymerase proteins which are directed to the nucleus of the infected cell through specific sequences present in the protein. For the NP this amino acid sequence has been found to be (single letter code) QLVWMA<sup>5</sup>CNSA<sup>10</sup>AFEDLRVLS (Davey et al., 1985, Cell 40: 667-675). Therefore, if it is desired to direct the foreign gene product to the nucleus (if by itself it would not ordinarily do so) the hybrid protein should be engineered to contain a domain which directs it there. This domain could be of influenza viral origin, but not necessarily so. Hybrid proteins can also be made from non-viral sources, as long as they contain the necessary sequences for replication by influenza virus (3' untranslated region, etc.). As another example, certain antigenic regions of the viral gene products may be substituted with foreign sequences. Townsend et al., (1985, Cell 42: 475-482), identified an epitope within the NP molecule which is able to elicit a vigorous CTL (cytotoxic T cell) response. This epitope spans residues 147-161 of the NP protein and consists of the amino acids TYQRTRQLVRLTGMDP. Substituting a short foreign epitope in place of this NP sequence may elicit a strong cellular immune response against the intact foreign antigen. Conversely, expression of a foreign gene product containing this 15 amino acid region may also help induce a strong cellular immune response against the foreign protein.

#### 5.1.5. Insertion of the Heterologous Gene Sequence into the HA or NA Gene Segments

The HA and NA proteins, coded for by separate gene segments, are the major surface glycoproteins of the virus. Consequently, these proteins are the major targets for the humoral immune response after infection. They have been the most widely-studied of all the influenza viral proteins as the three-dimensional structures of both these proteins have been solved.

The three-dimensional structure of the H3 hemagglutinin along with sequence information on large numbers of variants has allowed for the elucidation of the antigenic sites on the HA molecule (Webster et al., 1983, In Genetics Of Influenza Virus, P. Palese and D. W. Kingsbury, eds.,

Springer-Verlag, Vienna, pp. 127-160). These sites fall into four discrete non-overlapping regions on the surface of the HA. These regions are highly variable and have also been shown to be able to accept insertions and deletions. Therefore, substitution of these sites within HA (e.g., site A; amino acids 122-147 of the A/HK/68 HA) with a portion of a foreign protein may provide for a vigorous humoral response against this foreign peptide. In a different approach, the foreign peptide sequence may be inserted within the antigenic site without deleting any viral sequences. Expression products of such constructs may be useful in vaccines against the foreign antigen, and may indeed circumvent a problem discussed earlier, that of propagation of the recombinant virus in the vaccinated host. An intact HA molecule with a substitution only in antigenic sites may allow for HA function and thus allow for the construction of a viable virus. Therefore, this virus can be grown without the need for additional helper functions. Of course, the virus should be attenuated in other ways to avoid any danger of accidental escape.

Other hybrid constructions may be made to express proteins on the cell surface or enable them to be released from the cell. As a surface glycoprotein, the HA has an amino-terminal cleavable signal sequence necessary for transport to the cell surface, and a carboxy-terminal sequence necessary for membrane anchoring. In order to express an intact foreign protein on the cell surface it may be necessary to use these HA signals to create a hybrid protein. Alternatively, if only the transport signals are present and the membrane anchoring domain is absent, the protein may be excreted out of the cell.

In the case of the NA protein, the three-dimensional structure is known but the antigenic sites are spread out over the surface of the molecule and are overlapping. This indicates that if a sequence is inserted within the NA molecule and it is expressed on the outside surface of the NA it will be immunogenic. Additionally, as a surface glycoprotein, the NA exhibits two striking differences from the HA protein. Firstly, the NA does not contain a cleavable signal sequence; in fact, the amino-terminal signal sequence acts as a membrane anchoring domain. The consequence of this, and the second difference between the NA and HA, is that the NA is orientated with the amino-terminus in the membrane while the HA is orientated with the carboxy-terminus in the membrane. Therefore it may be advantageous in some cases to construct a hybrid NA protein, since the fusion protein will be orientated opposite of a HA-fusion hybrid.

#### 5.1.6. Insertion of the Heterologous Gene into the NS and M Gene Segments

The unique property of the NS and M segments as compared to the other six gene segments of influenza virus is that these segments code for at least two protein products. In each case, one protein is coded for by an mRNA which is co-linear with genomic RNA while the other protein is coded for by a spliced message. However, since the splice donor site occurs within the coding region for the co-linear transcript, the NS1 and NS2 proteins have an identical 10 amino acid amino terminus while MI and M2 have an identical 14 amino acid amino terminus.

As a result of this unique structure, recombinant viruses may be constructed so as to replace one gene product within the segment while leaving the second product intact. For instance, replacement of the bulk of the NS2 or M2 coding region with a foreign gene product (keeping the splice acceptor site) could result in the expression of an intact NS1

or M1 protein and a fusion protein instead of NS2 or M2. Alternatively, a foreign gene may be inserted within the NS gene segment without affecting either NS1 or NS2 expression. Although most NS genes contain a substantial overlap of NS1 and NS2 reading frames, certain natural NS genes do not. We have analyzed the NS gene segment from A/Ty/Or/71 virus (Norton et al., 1987, *Virology* 156: 204–213) and found that in this particular gene, the NS1 protein terminates at nucleotide position 409 of the NS gene segment while the splice acceptor site for the NS2 is at nucleotide position 528. Therefore, a foreign gene could be placed between the termination codon of the NS1 coding region and the splice acceptor site of the NS2 coding region without affecting either protein. It may be necessary to include a splice acceptor site at the 5' end of the foreign gene sequence to ensure protein production (this would encode a hybrid protein containing the amino-terminus of NS1). In this way, the recombinant virus should not be defective and should be able to be propagated without need of helper functions.

Although the influenza virus genome consists of eight functional gene segments it is unknown how many actual segments a virus packages. It has been suggested that influenza can package more than eight segments, and possibly up to 12 (Lamb and Choppin, 1983, *Ann. Rev. Biochem.* 52: 467–506). This would allow for easier propagation of recombinant virus in that "ninth" gene segment could be designed to express the foreign gene product. Although this "ninth" segment may be incorporated into some viruses, it would soon be lost during virus growth unless some selection is supplied. This can be accomplished by "uncoupling" the NS or M gene segment. The NS2 coding portion could be removed from the NS gene segment and placed on the gene segment coding for the foreign protein (along with appropriate splicing signals). Alternatively, a bicistronic mRNA could be constructed to permit internal initiation to "unsplice" these viral sequences; for example, using the sequences described by Pelletier et al., 1988, *Nature* 334: 320–325. The resulting recombinant virus with the "uncoupled" NS or M gene would be able to propagate on its own and also would necessarily have to package the "ninth" gene segment, thus ensuring expression of the foreign gene.

## 5.2. Expression of Heterologous Gene Products Using Recombinant RNA Template

The recombinant templates prepared as described above can be used in a variety of ways to express the heterologous gene products in appropriate host cells or to create chimeric viruses that express the heterologous gene products. In one embodiment, the recombinant template can be combined with viral polymerase complex purified as described in Section 6, *infra*, to produce rRNPs which are infectious. Alternatively, the recombinant template may be mixed with viral polymerase complex prepared using recombinant DNA methods (e.g., see Kingsbury et al., 1987, *Virology* 156: 396–403). Such rRNPs, when used to transfect appropriate host cells, may direct the expression of the heterologous gene product at high levels. Host cell systems which provide for high levels of expression include continuous cell lines that supply viral functions such as cell lines superinfected with influenza, cell lines engineered to complement influenza viral functions, etc.

In an alternate embodiment of the invention, the recombinant templates or the rRNPs may be used to transfect cell lines that express the viral polymerase proteins in order to achieve expression of the heterologous gene product. To this end, transformed cell lines that express all three polymerase

proteins such as 3P-38 and 3P-133 (Krystal et al., 1986, *Proc. Natl. Acad. Sci. U.S.A.* 83: 2709–2713) may be utilized as appropriate host cells. Host cells may be similarly engineered to provide other viral functions or additional functions such as NP.

### 5.2.1. Purification of the Viral Polymerase

The viral polymerase proteins used to produce the rRNPs may be purified from dissociated RNP cores isolated from whole virus. In general, RNP cores may be prepared using standard methods (Plotch et al., 1981, *Cell* 23: 847–858; Rochavansky, 1976, *Virology* 73: 327–338). The pooled RNP cores may then be centrifuged on a second gradient of CsCl (1.5–3.0M) and glycerol (30%–45%) as described by Honda et al., 1988, *J. Biochem.* 104: 1021–1026. The active viral polymerase fractions may be isolated from top of the gradient, i.e., in the region of the gradient correlating with 1.5 to 2.0M CsCl and corresponding to the fraction Honda et al. identified as "NP". Surprisingly, this fraction contains all the viral polymerase proteins required for the active complex. Moreover, the P proteins which may be recovered from the bottom of the gradient are not required, and indeed do not provide for the transcription of full length viral RNA. Thus, it appears that the so-called "NP" fraction contains, in addition to NP, the active forms of the PB2, PB1, and PA proteins.

### 5.2.2. High Concentrations of Polymerase are Required for Cap-Primed RNA Synthesis

High concentrations of viral polymerase complex are able to catalyze this virus-specific cap-endonuclease primed transcription. Under the conditions specified in Section 6 *infra*, about 50 ng NP with 200 pg of the three P proteins were found to react optimally with 5 to 10 ng RNA reaction. The observation has been that although the NP selectively encapsidates influenza vRNA or cRNA *in vivo*, the NP will bind to RNA nonspecifically *in vitro* (Kingsbury, et al., 1987, *Virology* 156: 396–403; Scholtissek and Becht, 1971, *J. Gen. Virol.* 10: 11–16). Presumably, in order for the viral polymerase to recognize the viral template RNAs in our *in vitro* reaction, they have to be encapsidated by the NP. Therefore, the addition of a capped mRNA primer would essentially compete with the template RNA for binding of NP. Since the dinucleotide ApG would not be expected to bind NP, the low concentration polymerase was able to use only the short templates with ApG. Supporting this hypothesis is the observation that the higher concentration polymerase preparation is inhibited through the addition of progressively higher amounts of either template RNA or any non-specific RNA. It should also be noted that the unusual specificity for the m7GpppXm cap 1 structure previously shown with viral RNPs was also found with the reconstituted RNPs.

### 5.2.3. Genomic Length RNA Templates are Efficiently Copied

Plasmid-derived RNA identical to segment 8 of the A/WSN/33 virus was specifically copied by the polymerase (using the PCR method described in FIG. 10). In reactions using RNA extracted from virus, all eight segments were copied, although the HA gene was copied at a lower level. The background in these reactions was decreased in comparison to the 30 to 53 nt templates, probably since the contaminating RNAs in the polymerase preparation were predominantly defective RNAs of small size. Recombinant templates encoding foreign genes transcribed in this system may be used to rescue the engineered gene in a virus particle.

## 5.3. Preparation of Chimeric Negative Strand RNA Virus

In order to prepare chimeric virus, reconstituted RNPs containing modified influenza virus RNAs or RNA coding



for foreign proteins may be used to transfect cells which are also infected with a "parent" influenza virus. Alternatively, the reconstituted RNP preparations may be mixed with the RNPs of wild type parent virus and used for transfection directly. Following reassortment, the novel viruses may be isolated and their genomes be identified through hybridization analysis. In additional approaches described herein for the production of infectious chimeric virus, rRNPs may be replicated in host cell systems that express the influenza viral polymerase proteins (e.g., in virus/host cell expression systems; transformed cell lines engineered to express the polymerase proteins, etc.), so that infectious chimeric virus are rescued; in this instance, helper virus need not be utilized since this function is provided by the viral polymerase proteins expressed. In a particularly desirable approach, cells infected with rRNPs engineered for all eight influenza virus segments may result in the production of infectious chimeric virus which contain the desired genotype; thus eliminating the need for a selection system.

Theoretically, one can replace any one of the eight gene segments, or part of any one of the eight segments with the foreign sequence. However, a necessary part of this equation is the ability to propagate the defective virus (defective because a normal viral gene product is missing or altered). A number of possible approaches exist to circumvent this problem. We have shown that mutants of influenza virus defective in the PB2 and NP proteins can be grown to substantially higher titers in cell lines which were constructed to constitutively express the polymerase and NP proteins (Krystal et al., 1986 Proc. Natl. Acad. Sci. U.S.A. 83: 2709-2813). Similar techniques may be used to construct transformed cell lines that constitutively express any of the influenza genes. These cell lines which are made to express the viral protein may be used to complement the defect in the recombinant virus and thereby propagate it. Alternatively, certain natural host range systems may be available to propagate recombinant virus. An example of this approach concerns the natural influenza isolate CR43-3. This virus will grow normally when passaged in primary chick kidney cells (PCK) but will not grow in Madin-Darby canine kidney cells (MDCK), a natural host for influenza (Maassab & DeBorde, 1983, Virology 130: 342-350). When we analyzed this virus we found that it codes for a defective NS1 protein caused by a deletion of 12 amino acids. The PCK cells contain some activity which either complements the defective NS1 protein or can completely substitute for the defective protein.

A third approach to propagating the recombinant virus may involve co-cultivation with wild-type virus. This could be done by simply taking recombinant virus and co-infecting cells with this and another wild-type virus (preferably a vaccine strain). The wild-type virus should complement for the defective virus gene product and allow growth of both the wild-type and recombinant virus. This would be an analogous situation to the propagation of defective-interfering particles of influenza virus (Nayak et al., 1983, In: Genetics of Influenza Viruses, P. Palese and D. W. Kingsbury, eds., Springer-Verlag, Vienna, pp. 255-279). In the case of defective-interfering viruses, conditions can be modified such that the majority of the propagated virus is the defective particle rather than the wild-type virus. Therefore this approach may be useful in generating high titer stocks of recombinant virus. However, these stocks would necessarily contain some wild-type virus.

Alternatively, synthetic RNPs may be replicated in cells co-infected with recombinant viruses that express the influenza virus polymerase proteins. In fact, this method may be

used to rescue recombinant infectious virus in accordance with the invention. To this end, the influenza virus polymerase proteins may be expressed in any expression vector/host cell system, including but not limited to viral expression vectors (e.g., vaccinia virus, adenovirus, baculovirus, etc.) or cell lines that express the polymerase proteins (e.g., see Krystal et al., 1986, Proc. Natl. Acad. Sci. USA 83: 2709-2713). Moreover, infection of host cells with rRNPs encoding all eight influenza virus proteins may result in the production of infectious chimeric virus particles. This system would eliminate the need for a selection system, as all recombinant virus produced would be of the desired genotype. In the examples herein, we describe a completely synthetic replication system where, rather than infecting cells with influenza virus, synthetic RNP's are replicated in cells through the action of influenza virus proteins expressed by recombinant vaccinia vectors. In this way we show that the only influenza virus proteins essential for transcription and replication of RNP are the three polymerase proteins and the nucleoprotein.

It should be noted that it may be possible to construct a recombinant virus without altering virus viability. These altered viruses would then be growth competent and would not need helper functions to replicate. For example, alterations in the hemagglutinin gene segment and the NS gene segment discussed, supra, may be used to construct such viable chimeric viruses.

In the examples infra, the construction of a recombinant plasmid is described that, following transcription by T7 polymerase, yielded an RNA template which was recognized and transcribed by the influenza virus polymerase in vitro. This RNA template corresponds to the NS RNA of an influenza virus except that the viral coding sequences are replaced by those of a CAT gene. This recombinant negative strand viral RNA template was then mixed with purified influenza virus polymerase to reconstitute an RNP complex. The recombinant RNP complex was transfected into cells which were then infected with influenza virus, leading to expression of CAT activity.

A number of factors indicate that this system represents a biologically active recombinant RNP complex which is under tight control of the signals for transcription, replication and packaging of influenza virus RNAs. First, the CAT gene is of negative polarity in the recombinant viral RNA used for RNP transfection. Thus, the incoming RNA cannot be translated directly in the cell and must first be transcribed by the influenza virus polymerase to permit translation and expression of the CAT gene. Secondly, neither transfected naked recombinant RNA alone in the presence of infecting helper virus, nor recombinant RNP complex in the absence of infecting helper virus is successful in inducing CAT activity. This suggests that influenza viral proteins provided by the incoming RNP, as well as by the infecting helper virus, are necessary for the amplification of the recombinant RNA template. Finally, after RNP-transfection and infection by helper virus, virus particles emerge which apparently contain the recombinant RNA, since these particles again induce CAT activity in freshly infected cells. These results suggest that the 26 3' terminal and the 22 5' terminal nucleotides corresponding to the terminal nucleotides in the influenza A virus NS RNA are sufficient to provide the signals for polymerase transcription and replication, as well as for packaging of the RNA into particles.

The foregoing results, which defined the cis acting sequences required for transcription, replication and packaging of influenza virus RNAs, were extended by additional working examples, described infra, which demonstrate that

recombinant DNA techniques can be used to introduce site-specific mutations into the genomes of infectious influenza viruses.

Synthetic RNAs, derived by transcription of plasmid RNA in vitro were used in RNP-transfection experiments to rescue infectious influenza virus. To enable selection of this virus, we chose a system that required the presence of a WSN-like neuraminidase gene in the rescued virus. Viruses containing this gene can grow in MDBK cells in the absence of protease in the medium (Schulman et al., 1977, *J. Virol.* 24: 170-176). The helper virus WSN-HK does not grow under these circumstances. Clearly, alternative selection systems exist. For example, antibody screens or conditionally lethal mutants could be used to isolate rescued viruses containing RNAs derived from plasmid DNAs. In the experiments viruses described infra, viruses which were WSN virus-like were recovered. The WSN NA gene was derived from plasmid DNAs or from purified WSN virion RNA (FIG. 17, lanes 2 and 5). In the latter case, using whole virion RNA for the RNP-transfection, we do not know whether other genes were also transferred to the rescued virus, since the helper virus shares the remaining seven genes with WSN virus. The rescued viruses had the expected RNA patterns (FIG. 17) and grew to titers in MDBK or MDCK cells which were indistinguishable from those of the wild type WSN virus. It should be noted that rescue of an NA RNA containing a single nucleotide deletion in the 5' nontranslated region was not possible. This again illustrates the importance of regulatory sequences present in the nontranslated regions of influenza virus RNAs. We also rescued virus using RNA that was engineered to contain 5 nucleotide changes in a 39 nucleotide long region (FIG. 16). We verified the presence of these mutations in the rescued mutant virus by direct sequencing of the RNA (FIG. 18). These mutations did not result in any amino acid change in the neuraminidase protein and thus were not expected to change the biological property of the virus. Although this virus was not extensively studied, its plaquing behavior and its growth characteristics were indistinguishable from that of wild type WSN virus. Using such technology, mutations may be introduced that will change the biological characteristics of influenza viruses. These studies will help in distinguishing the precise functions of all the viral proteins, including those of the nonstructural proteins. In addition, the nontranslated regions of the genome can be studied by mutagenesis, which should lead to a better understanding of the regulatory signals present in viral RNAs. An additional area of great interest concerns the development of the influenza virus system as a vaccine vector.

#### 5.4. Vaccine Formulations Using the Chimeric Viruses

Virtually any heterologous gene sequence may be constructed into the chimeric viruses of the invention for use in vaccines. Preferably, epitopes that induce a protective immune response to any of a variety of pathogens, or antigens that bind neutralizing antibodies may be expressed by or as part of the chimeric viruses. For example, heterologous gene sequences that can be constructed into the chimeric viruses of the invention for use in vaccines include but are not limited to epitopes of human immunodeficiency virus (HIV) such as gp120; hepatitis B virus surface antigen (HBsAg); the glycoproteins of herpes virus (e.g., gD, gE); VP1 of poliovirus; antigenic determinants of non-viral pathogens such as bacteria and parasites, to name but a few. In another embodiment, all or portions of immunoglobulin genes may be expressed. For example, variable regions of

anti-idiotypic immunoglobulins that mimic such epitopes may be constructed into the chimeric viruses of the invention.

Either a live recombinant viral vaccine or an inactivated recombinant viral vaccine can be formulated. A live vaccine may be preferred because multiplication in the host leads to a prolonged stimulus of similar kind and magnitude to that occurring in natural infections, and therefore, confers substantial, long-lasting immunity. Production of such live recombinant virus vaccine formulations may be accomplished using conventional methods involving propagation of the virus in cell culture or in the allantois of the chick embryo followed by purification.

In this regard, the use of genetically engineered influenza virus (vectors) for vaccine purposes may require the presence of attenuation characteristics in these strains. Current live virus vaccine candidates for use in humans are either cold adapted, temperature sensitive, or passaged so that they derive several (six) genes from avian viruses, which results in attenuation. The introduction of appropriate mutations (e.g., deletions) into the templates used for transfection may produce the novel viruses with attenuation characteristics. For example, specific missense mutations which are associated with temperature sensitivity or cold adaptation can be made into deletion mutations. These mutations should be more stable than the point mutations associated with cold or temperature sensitive mutants and reversion frequencies should be extremely low.

Alternatively, chimeric viruses with "suicide" characteristics may be constructed. Such viruses would go through only one or a few rounds of replication in the host. For example, cleavage of the HA is necessary to allow for reinitiation of replication. Therefore, changes in the HA cleavage site may provide a virus that replicates in an appropriate cell system but not in the human host. When used as a vaccine, the recombinant virus would go through a single replication cycle and induce a sufficient level of immune response but it would not go further in the human host and cause disease. Recombinant viruses lacking one or more of the essential influenza virus genes would not be able to undergo successive rounds of replication. Such defective viruses can be produced by co-transfecting reconstituted RNPs lacking a specific gene(s) into cell lines which permanently express this gene(s). Viruses lacking an essential gene(s) will be replicated in these cell lines but when administered to the human host will not be able to complete a round of replication. Such preparations may transcribe and translate—in this abortive cycle—a sufficient number of genes to induce an immune response. Alternatively, larger quantities of the strains could be administered, so that these preparations serve as inactivated (killed) virus vaccines. For inactivated vaccines, it is preferred that the heterologous gene product be expressed as a viral component, so that the gene product is associated with the virion. The advantage of such preparations is that they contain native proteins and do not undergo inactivation by treatment with formalin or other agents used in the manufacturing of killed virus vaccines.

In another embodiment of this aspect of the invention, inactivated vaccine formulations may be prepared using conventional techniques to "kill" the chimeric viruses. Inactivated vaccines are "dead" in the sense that their infectivity has been destroyed. Ideally, the infectivity of the virus is destroyed without affecting its immunogenicity. In order to prepare inactivated vaccines, the chimeric virus may be grown in cell culture or in the allantois of the chick embryo, purified by zonal ultracentrifugation, inactivated by formaldehyde or  $\beta$ -propiolactone, and pooled. The resulting vaccine is usually inoculated intramuscularly.



Inactivated viruses may be formulated with a suitable adjuvant in order to enhance the immunological response. Such adjuvants may include but are not limited to mineral gels, e.g., aluminum hydroxide; surface active substances such as lysolecithin, pluronic polyols, polyanions; peptides; oil emulsions; and potentially useful human adjuvants such as BCG and *Corynebacterium parvum*.

Many methods may be used to introduce the vaccine formulations described above. These include but are not limited to oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, and intranasal routes. It may be preferable to introduce the chimeric virus vaccine formulation via the natural route of infection of the pathogen for which the vaccine is designed. Where a live chimeric virus vaccine preparation is used, it may be preferable to introduce the formulation via the natural route of infection for influenza virus. The ability of influenza virus to induce a vigorous secretory and cellular immune response can be used advantageously. For example, infection of the respiratory tract by chimeric influenza viruses may induce a strong secretory immune response, for example in the urogenital system, with concomitant protection against a particular disease causing agent.

## 6. EXAMPLE: PROMOTER ANALYSIS OF THE INFLUENZA VIRAL RNA POLYMERASE

In the examples described below, polymerase which is depleted of genomic RNA was prepared from the upper fractions of the CsCl-glycerol gradient centrifugation. This polymerase is able to copy short model templates which are derived from transcription of appropriate plasmic DNA with bacteriophage T7 RNA polymerase in a sequence-specific manner. The termini of this model RNA are identical to the 3' 15 and 5' 22 nucleotides conserved in segment 8 from all influenza A viral RNAs. By manipulating the plasmid in order to prepare different RNAs to serve as template, we demonstrated that recognition of and synthesis from this model RNA was specific for the promoter at the 3' terminal sequence and did not require the panhandle. In addition, site specific mutagenesis identified nucleotide positions responsible for the viral polymerase favoring synthesis from genomic sense templates over complementary sense RNA. Conditions were also found in which cap-endonuclease primed RNA synthesis could be observed using model RNAs. In addition, the reconstituted system permitted virus-specific synthesis from genomic length RNAs, derived either from plasmids or from RNA purified from virus through phenol extraction.

### 6.1. Materials and Methods

#### 6.1.1. Purification of the Viral RNA Polymerase

RNP cores were prepared from whole virus using standard methods (Plotch, et al., 1981, Cell 23: 847-858; Rochavansky, 1976, Virology 73: 327-338). Two to three milligrams of virus were disrupted by incubating in 1.5% Triton N-101, 10 mg/ml lysolecithin, 100 mM tris-HCl, pH 8.0, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 5% glycerol and 1.5 mM dithiothreitol. The sample was fractionated by centrifugation on a 30-70% glycerol (w/v) step gradient in the presence of 50 mM tris-HCl, pH 7.8 and 150 mM NaCl. The core preparation was centrifuged at 45,000 rpm in an SW50.1 rotor for 4 hours at 4° C. Fractions enriched in RNP were identified by SDS-polyacrylamide gel electrophoresis of protein samples from each fraction and staining with silver. The core fractions were then subjected to a second gradient centrifugation as was described in Honda et al. 1988, J.

Biochem. 104: 1021-1026. This second gradient had steps of 0.5 ml 3.0M CsCl and 45% (w/v) glycerol, 1.75 ml 2.5 M CsCl and 40% glycerol, 1.25 ml 2.0M CsCl and 35% glycerol, and 1.0 ml of 1.5 M CsCl and 30% glycerol. All steps were buffered with 50 mM tris-HCl, pH 7.6 and 100 mM NaCl. 0.5 ml of RNP cores were layered on top and the sample was centrifuged at 45,000 rpm in an SW50.1 rotor for 25 hours at 4° C. Polymerase fractions were again identified by SDS-polyacrylamide electrophoresis of the protein samples and silver staining. Active polymerase fractions were generally found in the region of the gradient correlating with 1.5 to 2.0 M CsCl. These fractions were pooled and then dialyzed against 50 mM tri-HCl, pH 7.6, 100 mM NaCl and 10 mM MgCl<sub>2</sub>, and concentrated in centricon-10 tubes (Amicon) or fractions were dialyzed in bags against 50 mM tris-HCl, pH 7.6, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, and 50% glycerol.

#### 6.1.2. Preparation of Plasmid

The plasmid design is indicated in FIG. 2. Insert DNA for the pV-wt plasmid was prepared using an Applied Biosystems DNA synthesizer. The "top" strand was

```
5'- GAAGCTTAATACGACTCACTATAAGTAGAAACAAGGGTG
TTTTTTCATATCATTTAACTTCACCTGCTTTTGCTGAA
TTCATTCTCTGCAGG-3'.
```

The "bottom" strand was synthesized by primer-extension with

```
5'- CCTGCAGAAGAATGA-3'
```

as primer. The 95 bp DNA was digested with HindIII and PstI and purified by extraction with phenol/chloroform, ethanol precipitation, and passage over a NACS-prepack ion Exchange column (Bethesda Research Laboratories). This DNA was ligated into pUC-19 which had been digested with HindIII and PstI and then used to transform *E. coli* strain DH5-α which had been made competent using standard protocols. Bacteria were spread on agar plates containing X-gal and IPTG, and blue colonies were found to have the plasmid containing the predicted insert since the small insert conserved the lacZ reading frame and did not contain a termination codon. The pM-wt plasmid was prepared by a similar strategy except that both strands were chemically synthesized with the upper strand having the sequence

```
5'- GAAGCTTAATACGACTCACTATAAGCAAAAGCAGGGTGA
AGTTTAAATGATAT — GAAAAACACCCTTGTTTCTACTG
AATTCATTCTTCTGCAGG-3'.
```

The pV-d5' plasmid (FIG. 2) was prepared using the oligonucleotides

```
5'- AGCTTAATACGACTCACTATAAGATCTATTAACT — T
CACCTGCTTTTGCTGAATTCATTCTCTGA-3' and
```

```
5'- GAAGAATGAAT — TCAGCAAAAGCAGGGTGAAGTTTAA
TAGATCTTATAGTGAGTCGTATTA-3'.
```

The DNAs were annealed and ligated into the HindIII/PstI digested pUC-19 and white colonies were found to contain the correct plasmid because this insert resulted in a frame-shift in the lacZ gene. The point mutants were isolated following digestion of pV-d5' with BglII and PstI and ligation of the linearized plasmid with a single stranded oligonucleotide of mixed composition. Since BglII leaves a 5' extension and PstI a 3' extension, a single oligonucleotide was all that was necessary for ligation of insert. The host cell was then able to repair gaps caused by the lack of a

complementary oligonucleotide. Oligonucleotides were designed to repair the frameshift in the lacZ gene so that bacteria which contained mutant plasmids were selected by their blue color.

Plasmid pHgaNS, which was used to prepare an RNA identical to segment 8 of A/WSN/33, was prepared using the primers

5'- CCGAATTCTTAATACGACTCACTATAAGTAGAAACAAGG  
GTG-3' and

5'- CCTCTAGACGCTCGAGAGCAAAAGCAGGTG-3'

in a polymerase chain reaction off of a cDNA clone. The product was then cloned into the XbaI/EcoRI window of pUC19.

#### 6.1.3. Preparation of RNA Templates

Plasmid DNAs were digested with MboII or other appropriate endonucleases (see FIG. 2), and the linearized DNA was transcribed using the bacteriophage T7 RNA polymerase. Run-off RNA transcripts were treated with RNase-free DNase 1 and then the RNA was purified from the proteins and free nucleotides using Qiagen tip-5 ion exchange columns (Qiagen, Inc.). Following precipitation in ethanol, purified RNAs were resuspended in water and a sample was analyzed by electrophoresis and followed by silver staining of the polyacrylamide gel in order to quantify the yield of RNA.

#### 6.1.4. Influenza Viral Polymerase Reactions

In a 25  $\mu$ l total volume, about 30  $\mu$ g of nucleoprotein and 200 pg total of the three polymerase proteins were mixed with 10 ng of template RNA and the solution was made up to a final concentration of: 50 mM Hepes pH 7.9, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.05% NP-40, 0.4 mM adenylyl-(3'-5')-guanosyl (ApG) dinucleotide (Pharmacia), 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP and approximately 0.6  $\mu$ M  $\alpha$ -<sup>32</sup>P-UTP (40  $\mu$ Ci at 3000 Ci/mmol, New England Nuclear). Reactions were assembled on ice and then transferred to a 30° C. water bath for 90 minutes. Reactions were terminated by the addition of 0.18 ml ice-cold 0.3M sodium acetate/10 mM EDTA and were then extracted with phenol/chloroform (1:1 volume ratio). Following the first extraction, 15  $\mu$ g polyI-polyC RNA was added as carrier, and the sample was extracted again with phenol/chloroform. The samples were then extracted with ether and precipitated in ethanol. Following centrifugation, the RNA pellet was washed twice with 70% ethanol and then dried under vacuum.

In reactions using the high concentration polymerase, conditions were identical as above except that 20 ng of template RNA were added. In reactions using genomic length RNAs, the amount of polymerase used was doubled, 50 ng of template RNA was used, and the UTP concentration was raised to 2.6  $\mu$ M.

The RNA was resuspended in a dye mix containing 78% formamide, 10 mM EDTA, 0.1% xylene cyanol and 0.05% bromophenol blue. Typically, a sample from this RNA was electrophoresed on an 8% polyacrylamide gel in the absence of urea, and the remainder was denatured by heating to 100° C. for 1.5 minutes and an aliquot was loaded on an 8% polyacrylamide gel containing 7.7M urea. Gels were fixed by a two step procedure, first in 10% acetic acid, and then in 25% methanol/8% acetic acid. Gels were dried onto filter paper and then exposed to x-ray film.

When different RNAs were being tested for use as template, the different RNA preparations were always analyzed on polyacrylamide gels and stained with silver in order

that equal amounts of each template were used. To quantitate the amount of product, gels were exposed to x-ray film in the absence of an intensifying screen in order to improve the linearity of the densitometer readings. Autoradiographs were analyzed using a FB910 scanning densitometer (Fisher Biotech) and peaks were evaluated using computer software from Fisher Biotech.

#### 6.1.5. Nuclease Analysis of Reaction Products

For ribonuclease T1 analysis of the two principle RNA products, reaction products were analyzed by 8% polyacrylamide gel electrophoresis (without urea) and the gel was not treated with fixative. The wet gel was exposed to an x-ray film and the appropriate gel pieces were located and excised. The gel piece was crushed in 0.3 ml containing 10 mM tris pH 7.5, 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 1  $\mu$ g tRNA as carrier. The RNA diffused into this solution for 3 hours and then the gel was pelleted and the supernatant was made 0.3M in sodium acetate. The supernatant was then extracted twice in phenol/chloroform and once in ether and then precipitated in ethanol. The RNA pellet was resuspended in 5  $\mu$ l formamide, denatured in boiling water for 1.5 minutes and then diluted by the addition of 0.1 ml 10 mM tris-HCl, pH 7.5, and 1 mM EDTA. Ribonuclease T1 (50 units, Boehringer Mannheim Biochemicals) was added and the samples were incubated for 60 minutes at 37° C. V-wt and M-wt RNAs synthesized with T7 RNA polymerase in the presence of  $\alpha$ -<sup>32</sup>P-UTP were similarly digested with RNase T1. Reaction products were extracted in phenol/chloroform and precipitated in ethanol and then were analyzed on 20% polyacrylamide gels containing 7.7M urea.

Nuclease S1 analysis of reaction products was done on transcribed RNA by first terminating the standard polymerase reaction through the addition of S1 buffer to a volume of 0.2 ml with 0.26M NaCl, 0.05M sodium acetate, pH 4.6, and 4.5 mM zinc sulfate. The sample was divided into two 0.1 ml volumes and 100 units of S1 nuclease (Sigma Chemical Company) were added to one tube. The samples were incubated for 60 minutes at 37° C. Following the incubation, EDTA (10 mM final concentration) and 15  $\mu$ g polyI-polyC RNA was added and the sample was extracted with phenol/chloroform and precipitated in ethanol. The samples were then subjected to polyacrylamide gel electrophoresis.

## 6.2. Results

### 6.2. 1. Preparation of Influenza Viral RNA Polymerase and of Template RNA

RNP cores of influenza virus A/Puerto Rico/8/34 were prepared by disruption of virus in lysolecithin and Triton N-101 followed by glycerol gradient centrifugation (Rochavansky, 1976, Virology 73: 327-338). Fractions containing cores were then subjected to a second centrifugation in a CsCl-glycerol step gradient (Honda, et al., 1988, J. Biochem. 104: 1021-1026). Fractions containing the polymerase were identified by gel electrophoresis of samples followed by silver-staining. FIG. 1 shows the polymerase preparation after CsCl centrifugation. Bovine serum albumin (BSA) was added during dialysis to protect against protein loss. Densitometric scanning of lane 4 compared to known quantities of whole virus in lanes 1 and 2 allowed us to estimate that the proteins in lane 4 consist of 150 ng of NP and about 1 ng total of the three polymerase proteins. One fifth of the preparation used for this gel was used per reaction.

The overall design of the plasmids used to prepare template RNAs in this study is depicted in FIG. 2. The entire insert was prepared using oligonucleotides from a DNA

synthesizer which were then cloned into the polylinker of pUC19. The insert contained a truncated promoter sequence recognized by the bacteriophage T7 RNA polymerase (Studier and Dunn, 1983, Cold Spring Harbor Symposia on Quantitative Biology, XLVII, 999-1007) so that the first nucleotides synthesized were the terminal 22 nucleotides (nt) of the conserved sequence from the 5' end of the genome RNA. When the plasmid was cut with restriction endonuclease MboII (which cuts 7 bases upstream of its recognition site), the RNA which resulted from T7 RNA polymerase transcription ended with the terminal 3' nucleotides of the influenza viral sequence. Included in the sequence was the poly-U stretch adjacent to the 5' end of the conserved terminus which is thought to comprise at least part of the termination-polyadenylation signal (Robertson, et al., 1981, J. Virol. 38, 157-163). The total length of this model genomic RNA was 53 nt since a 16 nt spacer separated the terminal conserved sequences. The model RNA which contained both termini identical to those of vRNA was named V-wt. The RNA M-wt encoded the exact complementary strand of V-wt so that the termini match those of complementary RNA (cRNA). V-wt and M-wt were constructed to serve as models for influenza virus-specific vRNA and cRNA, respectively.

#### 6.2.2. Viral Polymerase Catalyzes Synthesis of a Full Length Copy of the Template

In the reaction using the influenza viral polymerase, V-wt template and ApG primer, a product was obtained which comigrated with a 53 nt RNA on denaturing gels. RNA migrating as a doublet at a position of about 40 to 45 nucleotides (FIG. 3A, lane 2) was also seen. This shorter product is shown below to be RNA which had terminated at a stretch of adenines present between nucleotides 43-48 in the virion sense template. In addition to the template specific transcripts, a general background of light bands could be seen which correspond to truncated RNA products transcribed from viral genomic RNA not removed during the CsCl-glycerol centrifugation step. When no primer is used, there was no specific transcription product seen (FIG. 3A, lane 3). Additional experiments showed globin mRNA, containing a terminal cap 1 structure, was inactive as primer using initial preparations of polymerase.

When the polymerase reaction was terminated by the addition of excess buffer favorable for nuclease S1 digestion and nuclease was added, the radioactively-labeled product was resistant to digestion (FIG. 3B, lane 2). By contrast these conditions very efficiently digested the V-wt single-stranded RNA radioactively synthesized with T7 RNA polymerase (FIG. 3B, lanes 3 and 4). These nuclease S1 data confirmed that the opposite strand was indeed being synthesized in these reactions. The product of the reaction might be a double stranded RNA, but it could not be ruled out that the product was in fact single stranded and later annealed to the template RNA in the presence of high salt used in the nuclease reaction.

The RNA products were purified by electrophoresis on an 8% gel, excised, eluted from the gel, and then digested by ribonuclease T1. Products were analyzed by electrophoresis and compared to the patterns generated by RNase T1 digestion of internally labeled M-wt and V-wt control probes. As can be seen in FIG. 3C, the full length RNA (lane 1) has the identical pattern as does the plus sense RNA, M-wt (lane 3), and it does not have the pattern of the V-wt RNA (lane 4). The observed patterns were essentially identical to that which is predicted from the sequence of the RNA and thus showed that the polymerase faithfully copied the V-wt template. The smaller RNA product, a doublet with most

templates, was also digested with RNase T1. Its pattern was similar to that of the full length RNA product (FIG. 3C, lane 2) except the 14 base oligonucleotide was not present. Instead, a faint 13 base oligonucleotide was seen, thus mapping the termination of the short RNA to position 44, a site where two uridines would be incorporated. Since the amount of smaller RNA product decreased at higher UTP concentrations and disappeared when CTP was used as label, these bands appeared to be an artifact of low UTP concentrations in the polymerase reaction.

#### 6.2.3. Conditions for the Polymerase Reactions Using Model RNA Templates

It was found that protein samples containing about 30 ng of NP protein and about 200 pg total of the three P proteins would react optimally with 5 to 10 ng of RNA. By using cold competitor RNA, polyI-polyC, it was found that excess RNA nonspecifically inhibited transcription, possibly via non-specific binding of the NP protein (Kingsbury, et al., 1987, Virology 156: 396-403; Scholtissek and Becht, 1971, J. Gen. Virol. 10: 11-16). In the absence of nonspecific competitor, variations in the amount of template between 1 and 10 ng produced little change in the efficiency of RNA synthesis. The NP protein and RNA were present at about equal molar concentrations and these were each about a thousand-fold in excess of the moles of the complex (assuming it to be 1:1:1) formed by the three P proteins in the typical reaction.

Since these reconstituted RNPs were able to use ApG but not globin mRNA as primer, we tested these model RNPs for other variables of the transcription reaction. In all other ways tested, the reconstituted RNPs behaved in solution similarly to those RNPs purified from detergent disrupted virus. The optimum temperature for RNA synthesis was 30° C. (FIG. 4A, lane 2) as has been repeatedly found for the viral polymerase (Bishop, et al., 1971, J. Virol. 8: 66-73; Takeuchi, et al., 1987, J. Biochem 101: 837-845; Ulmanen, et al., 1983, J. Virol. 45: 27-35). Also, the most active salt conditions were 60 mM NaCl (FIG. 4B, lane 2), again consistent with conditions used by several groups (Bishop, et al., 1971, J. Virol. 8: 66-73; Honda, et al., 1988, J. Biochem. 104: 1021-1026; Shapiro, and Krug, 1988, J. Virol. 62: 2285-2290). FIG. 4C shows a time-course experiment. The amount of RNA synthesis appeared to increase roughly linearly for the first 90 minutes, as was found for viral RNPs (Takeguchi, et al., 1987, J. Biochem. 101: 837-845).

#### 6.2.4. Specificity of the Elongation Reaction

Various RNAs were tested for suitability as templates for the RNA polymerase of influenza virus. The pV-wt plasmid clone was digested with either EcoRI, PstI or SmaI, and T7 polymerase was used to transcribe RNA. This resulted in RNAs identical to V-wt except for the addition of 5, 13 and 38 nt at the 3' end. In FIG. 5A an overexposure of an autoradiograph is shown in order to demonstrate that no transcripts over background were observed in reactions which contained as template: two of the RNAs identical to V-wt except they contained 13 and 38 nt of extra sequence on the 3' terminus (lanes 1 and 2); a single stranded DNA of identical sequence to that of V-wt (lane 4); and an unrelated 80 nt RNA generated by transcribing the polylinker of pIBI-31 with T3 RNA polymerase (lane 5). However, the V-Eco template, containing five extra nucleotides on the 3' end, could be recognized and faithfully transcribed, although at approximately one-third the efficiency of the wild type V-wt RNA (FIG. 5B, lane 3). It is interesting to note that initiation on the V-Eco RNA by the influenza viral poly-

merase appeared to occur at the correct base since the transcribed RNA was the same size as the product from the V-wt template.

#### 6.2.5. Analysis of the Promoter Region for the Viral RNA Polymerase

The original construct used for these studies contained the sequences of both RNA termini of genomic RNAs which could base pair and thus form a panhandle. This was done since it was shown that the vRNA in virions and in RNPs in infected cells was in circular conformation via the 15 to 16 nt long panhandle (Honda, et al., 1988, J. Biochem. 104: 1021-1026; Hsu, et al., 1987, Proc. Natl. Acad. Sci. USA 84: 8140-8144). It was further shown that the viral polymerase was bound to the double stranded structure (Honda, et al., 1988, J. Biochem. 104: 1021-1026), thus leading to the suggestion that the promoter for RNA synthesis was the panhandle. In order to test whether the panhandle was an absolute requirement for recognition, the following templates were used: the plasmid pV-wt was digested with DraI prior to transcription by the T7 polymerase (FIG. 2). This should result in an RNA molecule of 32 nt containing only virus-specific sequences from the 5' end of the RNA. When this RNA was used as template, no apparent product was produced (FIG. 5B, lane 2). Therefore the 3' terminus of virion RNA was required for this reaction. This finding was consistent with the fact that the initiation site at the 3' end of V-wt was not present in V-Dra. A second plasmid clone was produced which deleted the 5' terminal sequences but kept intact the 3' terminus. This clone, pV-d5', when digested with MboII and used for transcription by T7 polymerase produced a major transcript of 30 nt and minor species of 29 and 31 nt. Surprisingly, this template was recognized and copied by the influenza viral polymerase. FIG. 7, lane 1, shows that the product of the viral RNA polymerase reaction with V-d5' contains multiple bands reflecting the input RNA. When the products shown in FIG. 7, lane 1, were eluted from gels and subjected to RNase T1 analysis, the pattern expected of the transcription product of V-d5' was observed. Since the V-d5' RNA template was copied, the panhandle was not required for viral polymerase binding and synthesis.

Although the 5' terminus was not required for synthesis by the polymerase, a distinct possibility was that V-wt RNA might be a preferred template as compared to V-d5'. In order to examine this, reactions were done in which the templates were mixed. The V-wt RNA was present at 5 ng in each reaction. The V-d5' was absent (FIG. 6, lane 1) or was present at a 1/5 molar ratio (FIG. 6, lane 2) or a 1/1 molar ratio (FIG. 6, lane 3). The relative intensities of the bands from each RNA were determined by densitometry of the autoradiograph. The values were corrected for the amount of the radioactive nucleotide, UTP, which could be incorporated into each product, and the value was normalized so that the level of synthesis in each lane was set equal to one. The level of copying of V-wt decreased as V-d5' was increased. When V-d5' was present in one fifth molar ratio, its corrected level of synthesis was about one fourth of that from V-wt (FIG. 6, lane 2). When the two templates were present in equimolar amounts, the level of synthesis from V-wt was about 60% of the total (FIG. 6, lane 3) which might be within the expected range of experimental error for equivalent levels of synthesis. Similar results were obtained when V-d5' RNA was kept constant and the V-wt RNA was varied. It was thus concluded that the panhandle-containing V-wt RNA was not greatly favored over the template RNA which only contained the proper 3' terminus.

#### 6.2.6. The Viral Polymerase Does Not Copy RNA Templates Containing Plus-Sense Termini

As described earlier, the influenza RNA polymerase performs three distinct activities during the course of an infection. Two activities involve the transcription of genome sense RNA and the third involves copying of the complementary sense RNA into vRNA. We therefore constructed an RNA template which contained the 5' and 3' termini of the complementary sense RNA of segment 8 (M-wt; FIG. 2).

When the M-wt RNA was used as template, little synthesis was observed (FIG. 5B, lane 4). In two experiments used for quantitation, the average level of synthesis from M-wt RNA was 4% that of V-wt. In comparing the V-wt and M-wt RNA promoters, the M-wt has only three transition changes and one point insertion within the 3' 15 nucleotides. These include a G to A change at position 3, a U to C change at position 5, a C to U change at position 8 and an inserted U between the ninth and tenth nucleotides (see Table II, below). In order to determine which of the four point differences in the 3' termini were responsible for the specificity, many combinations of these were prepared and assayed for efficiency as a template (FIG. 7). These templates were derivatives of V-d5' since they did not contain the 5' terminus. The results of densitometry scans of several experiments are outlined in Table II.

TABLE II

QUANTITATIVE COMPARISON OF THE EFFECT OF  
POINT MUTATIONS IN THE PROMOTER SEQUENCE\*

Template	3' sequence	Level of RNA Synthesis
V-d5'	CACCCUGCUUUUGCU-OH	1
V-A3	CACCCUGCUUUUACU-OH	0.4
V-C5	CACCCUGCUUUGCU-OH	1.0
V-dU <sub>25</sub> U <sub>8</sub>	CACCCUGUUUUUGCU-OH	1.0
V-U <sub>8</sub> A <sub>3</sub>	CACCCUGUUUUUACU-OH	0.08
V-U <sub>8</sub> C <sub>5</sub>	CACCCUGUUUUGCU-OH	0.3
V-iU <sub>10</sub>	CACCCUUGCUUUUGCU-OH	0.7
V-iU <sub>10</sub> A <sub>3</sub>	CACCCUUGCUUUUACU-OH	0.06
V-iU <sub>10</sub> U <sub>8</sub> A <sub>3</sub>	CACCCUUGUUUUUACU-OH	0.2
V-iU <sub>10</sub> U <sub>8</sub> C <sub>5</sub> A <sub>3</sub>	CACCCUUGUUUCUACU-OH	0.2

\*Sequences of V-wt, M-wt and V-d5' are shown in FIG. 2. All other RNAs are identical to V-d5' except for the indicated positions. The subscripted number indicates the distance from the 3' end of a change, and d and i refer to deleted or inserted nucleotides.

As shown in Table II, single point changes in V-d5' were equally well copied as compared to V-d5' itself, except for the V-A<sub>3</sub> RNA which was copied at 40% efficiency (FIG. 7, lane 10; Table II). When RNAs with two changes were tested, the activity generally dropped to very low levels (FIG. 7, lanes 3, 4, and 5). Therefore, these experiments confirmed that the specificity of the reactions for V-wt over M-wt was the result of the combination of the nucleotide changes present at the 3' terminus of M-wt.

#### 6.2.7. Cap-Endonuclease Primed RNA Synthesis

The method of purifying the viral polymerase was modified in order to decrease loss of protein during dialysis. Rather than using the Amicon centricon-10 dialysis system, the enzyme was dialyzed in standard membranes resulting in higher concentrations of all four viral core proteins. The pattern of the protein gel of this preparation was identical to that shown in FIG. 1, lane 4, except that there is no BSA-derived band. It was found that 5 µl of this preparation, containing 150 ng of NP and 5 ng total of the three polymerase proteins, reacted optimally with 10 to 40 ng of model RNA template. However, the use of higher levels of protein increased the background, possibly due to higher levels of contaminating RNAs (virion RNAs not removed by CsCl centrifugation) yielding products of the size class

around 50–75 nt, complicating analysis of RNA templates containing a length of 50 nt.

This high concentration polymerase preparation was now active in cap-endonuclease primed RNA synthesis (FIG. 8A, lane 4) and also in primer-independent replication of the template RNA (FIG. 8A, lane 2). When globin mRNA was used as primer for transcription from the 30 nt V-ds' template, a triplet of bands of size about 42 to 44 nt was apparent as product (FIG. 8A, lane 4), consistent with cleavage of the cap structure at about 12 nt from the 5' end of the mRNA and use of this oligonucleotide to initiate synthesis from the 30 nt model template. Since excess RNA inhibits RNA synthesis, probably via nonspecific binding of NP *in vitro* as discussed above, the optimal amount of cap donor RNA added to each reaction was found to be 100 ng, which is much lower than is usually used with preformed RNP structures (e.g., Bouloy, et al., 1980, Proc. Natl. Acad. Sci. USA 77: 3952–3956). The most effective primer was ApG (FIG. 8A, lane 5 and lighter exposure in lane 6). The product migrates slower than that of the input template (FIG. 8A, lane 1) or the product in the absence of primer (FIG. 8A, lane 2) probably since the 5' terminus of the ApG product is unphosphorylated. The intensity of the ApG-primed product was about ten-fold higher than that of the cap-primed product, but at 0.4 mM, ApG was at a 60,000-fold molar excess of the concentration of the cap donors. Thus, although the intensity of the product band from cap-priming was about ten-fold lower than that from ApG priming, the cap-primed reaction was about 6000-fold more efficient on a molar basis. This value is similar to the approximately 4000-fold excess efficiency observed previously for the viral polymerase (Bouloy, et al., 1980, Proc. Natl. Acad. Sci. USA 77: 3952–3956). It has been previously shown that cap donor RNAs containing a cap 0 structure, as in BMV RNA, are about ten-fold less active in priming the influenza viral polymerase (Bouloy, et al., 1980, Proc. Natl. Acad. Sci. USA 77: 3952–3956). This unusual cap specificity was shared by the reconstituted RNPs studied here as the specific product from the model RNA was greatly decreased in reactions containing BMV RNA as cap donor. A 30 nt product was observed in lanes 2–4, probably due to primerless replication of the model template.

That the product RNAs were of the opposite sense of the input template V-ds' was shown by nuclease S1 analysis (FIG. 8B). The ApG-primed (FIG. 8B, lanes 1 and 2) and the primerless (FIG. 8B, lanes 3 and 4) RNA products were essentially nuclease resistant. The product of the cap-primed reaction (FIG. 8B, lanes 5 and 6) was partially sensitive to nuclease as about 12 nt were digested from the product. These results were most consistent with the 5' 12 nt being of mRNA origin as has been shown many times for influenza virus-specific mRNA synthesis.

The promoter specificity of this polymerase preparation in reactions primed with ApG was found to be essentially identical to those for the lower concentration enzyme as shown earlier. However, attempts thus far to perform similar analyses of promoter specificity with the primerless and cap-primed reactions have been frustrated by the comparatively high levels of background, thus making quantitation difficult.

#### 6.2.8. Replication of Genomic Length RNA Templates

A full-length 890 nt RNA identical to the sequence of A/WSN/33 segment 8 was prepared by T7 RNA polymerase transcription of plasmid DNA, pHgaNS, which had been digested with restriction endonuclease HgaI. This RNA was copied in ApG-primed reactions containing 10  $\mu$ l of the high

concentration polymerase (FIG. 9, lane 8). That the RNA was in fact a copy of the template was demonstrated by its resistance to nuclease S1 (FIG. 9, lane 9). A similar product was observed in the absence of primer (FIG. 9, lanes 2 and 3). Confirmation that these product RNAs were full length copies of the template was done by RNase T1 analysis. Virion RNA purified from phenol-extracted A/PR/8/34 virus was similarly copied in ApG primed reaction (FIG. 9, lanes 10 and 11) and in the absence of primer (FIG. 9, lanes 4 and 5). Interestingly, the product from replication of the HA gene was at greatly reduced levels. The 3' end of this RNA differs from that of segment 8 only at nucleotides 14 and 15, suggesting importance for these nucleotides in the promoter for RNA synthesis. In addition, we found that when whole viral RNA was used in the reconstituted RNPs, the level of acid precipitable counts was about 70% of that observed with native RNPs. The viral polymerase was also able to copy these full length RNAs when globin mRNA was used in cap-primed reaction.

### 7. EXAMPLE: EXPRESSION AND PACKAGING OF A FOREIGN GENE BY RECOMBINANT INFLUENZA VIRUS

The expression of the chloramphenicol transferase gene (CAT) using rRNPs is described. The rRNPs were prepared using pIVACAT (originally referred to as pCATcNS), a recombinant plasmid containing the CAT gene. The pIVACAT plasmid is a pUC19 plasmid containing in sequence: the T7-promoter; the 5'-(viral-sense) noncoding flanking sequence of the influenza A/PR8/34 RNA segment 8 (encodes the NS proteins); a BglII cloning site; the complete coding sequence of the chloramphenicol transferase (CAT) gene in the reversed and complemented order; the 3'-(viral-sense) noncoding NS RNA sequence;

and several restriction sites allowing run-off transcription of the template. The pIVACAT can be transcribed using T7 polymerase to create an RNA with influenza A viral-sense flanking sequences around a CAT gene in reversed orientation.

The *in vivo* experiments described in the subsections below utilized the recombinant RNA molecule described containing sequences corresponding to the untranslated 3' and 5' terminal sequences of the NS RNA of influenza virus A/PR8/34 flanking the antisense-oriented open reading frame of the CAT gene. This RNA was mixed with purified influenza virus polymerase complex and transfected into MDCK (or 293) cells. Following infection with influenza A/WSN/33 virus, CAT activity was measured in the RNP-transfected cells and amplification of the gene was indicated. In addition, the recombinant influenza virus gene was packaged into virus particles, since CAT activity was demonstrated in cells following infection with the recombinant virus preparation.

#### 7.1. Materials and Methods

In order to get the flanking sequences of the NS RNA fused to the coding sequence of the CAT gene, the following strategy was used. Two suitable internal restriction sites were selected, close to the start and stop codon of the CAT gene, that would allow the replacement of the sequences flanking the CAT gene in the pCM7 plasmid with the 3'- and 5'- NS RNA sequences. At the 5' end, a SfaNI site was chosen (which generates a cut 57 nt from the ATG), and at the 3' end a ScaI site which generates a cut 28 nt from the end of the gene (stop codon included). Next, four synthetic oligonucleotides were made using an Applied Biosystems



0.5% DMSO in PBS/gelatine (0.1 mg/ml gelatine) for 30 minutes at room temperature. After removal of this solution, 200  $\mu$ g of  $\mu$ l PBS/gelatine containing 1  $\mu$ g IVACAT 1 RNA (1–2  $\mu$ l), 20  $\mu$ l of the purified polymerase preparation and 4  $\mu$ l of Rnasin was added to the cells and incubated for 1 hour at 37° C. This was followed by the addition of gradient purified influenza A/WSN/33 virus (moi 2–10). After incubation for one hour at 37° C., 2.5 ml of either DMEM+10% FCS media (293 cells) or MEM media (MDCK cells) was added. In some experiments MDCK cells were first infected and subsequently RNP-transfected. Harvesting of cells was done in NET buffer or in media, using a rubber policeman (MDCK cells), or by gentle suspension (293 cells). Cells were spun down and the pellets were resuspended in 100  $\mu$ l of 0.25 M Tris buffer, pH 7.5. The samples were subsequently freeze-thawed three-times and the cell debris was pelleted. The supernatant was used for CAT assays.

#### 7.1.6. Passaging of Virus from RNP-Transfected Cells

MDCK cells were infected with helper virus and RNP-transfected 2 hours later as described above. After 1 hour cells and media were collected and cells were spun down. 100  $\mu$ l of the supernatant media, containing virus, was added to 35 mm dishes with MDCK cells. After 12 hours these cells and media were collected and assayed for CAT activity. Virus contained in this supernatant media was used for subsequent rounds of infection of MDCK cells in 35 mm dishes.

#### 7.1.7. CAT Assays

CAT assays were done according to standard procedures, adapted from Gorman et al., 1982, Mol. Cell. Biol. 2: 1044–1051. The assays contained 10  $\mu$ l of <sup>14</sup>C chloramphenicol (0.5  $\mu$ Ci; 8.3 nM; NEN), 20  $\mu$ l of 40 mM acetyl CoA (Boehringer) and 50  $\mu$ l of cell extracts in 0.25M Tris buffer (pH 7.5). Incubation times were 16–18 hours.

### 7.2. Results

rRNA templates were prepared from HgaI digested, end filled linearized pCATcNS using the bacteriophage T7 RNA polymerase as described in Section 6. The rRNA templates were combined with the viral RNA polymerase complex prepared as described in Section 6.1.1., and the resulting rRNPs were used to transfect MDCK and 293 cells lines which were superinfected with influenza A/WSN33. In each cell line transfected with the rRNPs, high levels of expression of CAT was obtained 6 hours post-infection. In addition, virus stocks obtained 24 hours post-infection synthesized high levels of CAT enzyme after subsequent passage in MDCK cells. The CAT-RNP was packaged into virus particles.

#### 7.2.1. Synthesis of IVACAT1 Template RNA

In order to study the transcription and replication signals of influenza A virus RNAs in vivo, we constructed plasmid pIVACAT1 (FIG. II) which directs the synthesis of an NS RNA-like transcript. This RNA shares the 22 5' terminal and the 26 3' terminal nucleotides with the NS RNA of influenza A/PR/8/34 virus and contain—instead of the coding sequences for the NS1 and NS2 proteins—those for a full-length CAT protein. For cloning purposes it also contains eight additional nucleotides including a BglII site between the stop codon of the CAT gene and the stretch of U's in the 5' noncoding region. The 17 promoter adjacent to the 5' noncoding sequences and the HgaI site downstream of the 3' end allow for the exact tailoring of the 5' and 3' ends. Run-off transcription using T7 polymerase generates a 716 nt long RNA: FIG. 12, lanes 2–4 show that this RNA is of discrete length and shorter than the 890 nt long marker NS RNA, which was synthesized by T7 transcription of pHgaNS (lane 1).

#### 7.2.2. The IVACAT1 RNA is Transcribed In Vitro by the Influenza A Virus RNA Polymerase

In the examples described in Section 6, it was demonstrated that synthetic RNAs containing at the 3' end the 15 3' terminal nucleotides of influenza virus RNA segment 8 can be transcribed in vitro using purified influenza A virus RNA polymerase. We tested whether unlabeled IVACAT1 RNA could be transcribed in a similar way. FIG. 12 lane 5 shows that the in vitro transcription reaction generated an RNA of discrete length and similar size to the product of the T7 transcription reaction suggesting synthesis of a full length product.

#### 7.2.3. RNP-Transfection and CAT Activity

Since the recombinant CAT RNA could be transcribed in vitro, a system was designed to test whether this RNA can be recognized and replicated in vivo (FIG. 13). Recombinant RNA was mixed with the purified polymerase to allow formation of viral RNP-like particles. To facilitate the association, the RNA/polymerase mixture was incubated in transcription buffer without nucleotides for 30 minutes at 30° C. prior to RNP-transfection. In some experiments, this preincubation step was omitted. RNP-transfections were either preceded or followed by infection with influenza A/WSN/33 virus, since the production of viral polymerase protein was expected to be necessary for efficient amplification of the gene. The cells used were either MDCK cells, which are readily susceptible to influenza A/WSN/33 virus infection, or human 293 cells, which support infection at a slower rate.

In order to determine whether the minus sense IVACAT1 RNA could be amplified and transcribed in vivo, an experiment was performed in 293 cells. Cells were transfected with RNP, virus infected one hour later and harvested at various times post-infection. FIG. 14A shows that at early times post infection only background levels of CAT activity were detected (lanes 5, 7 and 9). However, significant levels of CAT activity appeared seven hours after virus infection (lane 11) A similar level of CAT activity was detected two hours later (lane 13). There were background levels of CAT activity in the mock transfected 5 cells at any time point (lanes 6, 8, 10, 12 and 14), and in control cells not infected with A/WSN/33 virus (lanes 1–4).

Preincubation of RNA and polymerase complex was not necessary for successful RNP-transfection. As can be seen in FIG. 14B, lanes 2 and 3, preincubation might actually cause a decrease in CAT activity, presumably due to RNA degradation during preincubation. In another control experiment, infection by helper virus of RNP-transfected cells was omitted (FIG. 14B, lanes 4 and 5). Since these lanes show no CAT activity we conclude that the IVACAT1 RNA is amplified specifically by the protein machinery supplied by the helper virus. In an additional control experiment, naked RNA was transfected into cells which were subsequently helper-infected or mock-infected. Again, no CAT activity was detected in these samples (FIG. 14B, lanes 6–9). Finally virus-infected cells which were not transfected with recombinant CAT-RNP also did not exhibit endogenous acetylation activity (FIG. 14B, lane 10). It thus appears that addition of the purified polymerase to the recombinant RNA as well as infection of cells by helper virus is important for successful expression of the CAT enzyme.

Experiments were also performed using MDCK cells, the usual tissue culture host cell for influenza virus (FIG. 14C). When the reconstituted recombinant CAT-RNP complex was transfected 1 hour before virus infection, little CAT activity was observed at 7 hours post virus infection (FIG.



14C, lane 1). When RNP-transfection was accomplished 2 hours after virus infection, expression of CAT was greatly enhanced at 7 hours post-virus infection (FIG. 14C, lane 3). Therefore, MDCK cells are also viable host cells for these experiments.

#### 7.2.4. The CAT-RNP is Packaged into Virus Particles

Since the recombinant CAT RNA can be replicated in vivo via helper virus functions, we examined whether virus produced in RNP-transfected and helper virus infected cells contained the CAT gene. MDCK cells were used in the experiment because they yield higher titers of infectious virus than 293 cells. MDCK cells were infected with A/WSN/33 virus, RNP-transfected 2 hours later and allowed to incubate overnight. At 14 hours post infection, media was harvested and cells were pelleted. Virus supernatant was then used to infect new MDCK cell monolayers. The inoculum was removed after 1 hour and cells were harvested at 12 hours post infection and assayed for CAT activity. FIG. 15 reveals that the virus preparation induces a level of CAT activity (lanes 2 and 3) which is significantly above control (lane 1). In this case, the addition of helper virus to the inoculum did not increase CAT activity (lane 4). Further passaging of supernatant virus on fresh MDCK cells did not result in measurable induction of CAT activity. This is not surprising as there is no selective pressure for retaining the CAT gene in these viral preparations. We excluded the possibility that we were transferring the original RNA/polymerase complex by pretreating the inocula with RNase. This treatment destroys viral RNPs of influenza virus (Pons et al., 1969, *Virology* 39: 250-259; Scholtissek and Becht, 1971 *J. Gen. Virol.* 10: 11-16).

### 8. RESCUE OF INFECTIOUS INFLUENZA VIRUSES USING RNA DERIVED FROM SPECIFIC RECOMBINANT DNAs

The experiments described in the subsections below demonstrate the rescue of infectious influenza viruses using RNA which is derived from specific recombinant DNAs. RNAs corresponding to the neuraminidase (NA) gene of influenza A/WSN/33 virus (WSN virus) were transcribed in vitro from appropriate plasmid DNAs and—following the addition of purified influenza virus polymerase complex (as described in Section 6.1.1. supra)—were transfected into MDBK cells as described in Section 7, supra. Superinfection with helper virus, lacking the WSN NA gene, resulted in the release of viruses containing the WSN NA gene. Thus, this technology allows the engineering of infectious influenza viruses using cDNA clones and site-specific mutagenesis of their genomes. Furthermore, this technology may allow for the construction of infectious chimeric influenza viruses which can be used as efficient vectors for gene expression in tissue culture, animals or man.

The experiments described in Sections 6 and 7 supra, demonstrate that the 15 3' terminal nucleotides of negative

virus downstream of a truncated T3 promoter (FIG. 16). Therefore, runoff transcription of this plasmid, cut at the Ksp6321 site, yields an RNA which is identical to the true genomic NA gene of the WSN virus (FIG. 17, lane 3). This RNA was then incubated with purified polymerase (purified as described in Section 6.1.1) and used in a ribonucleoprotein (RNP) transfection experiment to allow the rescue of infectious virus using helper virus which did not contain the WSN virus NA. The choice of WSN-HK helper virus was based on the need for a strong selection system by which to isolate a rescued virus. Previously, it was shown that the WSN-HK virus can only form plaques in MDBK cells when protease is added to the medium. This is in marked contrast to WSN virus (isogenic to WSN-HK virus except for the neuraminidase gene), which in the absence of protease readily replicates in MDBK cells and forms large, easily visible plaques (Schulman et al., 1977, *J. Virol.* 24: 170-176).

## 8.1. Materials and Methods

### 8.1.1. Viruses and Cells

Influenza A/WSN/33 virus and A/WSN-HK virus were grown in Madin-Darby canine kidney (MDCK) cells and embryonated eggs, respectively (Sugiura et al., 1972, *J. Virol.* 10: 639-647; Schulman et al., 1977, *J. Virol.* 24: 170-176). Influenza A/PR/8/34 virus was also grown in embryonated eggs. Madin-Darby bovine kidney (MDBK) cells were used for the transfection experiments and for selection of rescued virus (Sugiura et al., 1972, *J. Virol.* 10: 639-647).

### 8.1.2. Construction of Plasmids

The pT3NAv, pT3NAv mut 1 and pT3NAv mut 2 plasmids were constructed by PCR-directed mutagenesis using a cloned copy of the WSN NA gene, which was obtained following standard procedures (Buonagurio et al., 1986, *Science* 232: 980-982). To construct pT3NAv, the following primers were used:

5'-CGGAATTCCTCTTCGAGCGAAAGCAGGAGTT-3' and

5'-CCAAGCTTATTAAACCCCTCACTAAAAGTAGAAACAAGGAGTT-3'.

After 35 cycles in a thermal cycler (Coy Lab Products, Mich.), the PCR product was digested with EcoRI and HindIII and cloned into pUC19. Plasmid pT3NAv mut 1 was constructed in a similar fashion except that the sequence of the primer was altered (FIG. 16). Plasmid pT3NAv mut 2 was constructed by cassette mutagenesis through the digestion of pT3NAv with PstI and NcoI and religation in the presence of the synthetic oligonucleotides—

5'-CATGGGTGAGTTTCGACCAAAATCTAGATTATAAAATAGGATACATATGCA-3'

and 5'-AATGTATCCTATTTTATAATCTAGATTTCGGTCGAAACTCACC-3'.

strand influenza virus RNAs are sufficient to allow transfection in vitro using purified influenza virus polymerase proteins. In addition, the studies using the reporter gene chloramphenicol acetyltransferase (CAT) show that the 22 5' terminal and the 26 3' terminal nucleotides of the viral RNAs contain all the signals necessary for transcription, replication and packaging of influenza virus RNAs. As an extension of these results, a plasmid, pT3NAv, was constructed which contained the complete NA gene of influenza A/WSN/33

Oligonucleotides were synthesized on an applied Biosystems DNA synthesizer. The final clones pT3NAv, pT3NAv mut 1 and pT3NAv mut 2 were grown up and the DNAs were partially sequenced starting from the flanking pUC 19 sequences and reaching into the coding sequences of the NA gene. The mutations in pT3NAv mut 2 were also confirmed by sequencing.

### 8.1.3. Purification of Influenza A Virus Polymerase and RNP Transfection in MDBK Cells



The RNA polymerase complex was purified from influenza A/PR/8/34 virus as described in Section 6. 1. 1, supra, and was then used for RNP transfection in MDBK cells using the protocol described in Section 7, supra, except that WSN-HK virus was used as helper virus at an moi of 1. RNAs used for RNP transfection were obtained by phenol extraction of purified virus or by transcription (using T3 polymerase) of pT3NAv, pT3NAv mut 1 and pT3NAv mut 2. All plasmids were digested with Ksp632I, end-filled by Klenow enzyme (BRL) and then transcribed in a runoff reaction as described in Section 7, supra.

## 8.2. Results

### 8.2.1. Rescue of Infectious Influenza Virus in MDBK Cells Using RNA Derived from Recombinant Plasmid DNA

A plasmid, pT3NAv, was constructed to contain the complete NA gene of influenza WSN virus downstream of a truncated T3 promoter (FIG. 16). Runoff transcription of the plasmid, cut at the Ksp632I site, yields an RNA which is identical in length to the true genomic NA gene of the WSN virus (FIG. 17, lane 3). This RNA was then incubated with purified polymerase and used in a ribonucleoprotein (RNP) transfection experiment to allow the rescue of infectious virus using helper virus. The choice of WSN-HK virus as helper virus was based on the need for a strong selection system by which to isolate a rescued virus. Previously, it was shown that the WSN-HK virus can only form plaques in MDBK cells when protease is added to the medium (Schulman et al., 1977, J. Virol. 24: 170-176). This is in marked contrast to WSN virus (isogenic to WSN-HK helper virus except for the neuraminidase gene), which in the absence of protease readily replicates in MDBK cells and forms large, easily visible plaques (Sugiura et al., 1972, J. Virol. 10: 639-647). MDBK cells were first infected with the WSN-HK helper virus and RNP-transfected one hour after virus infection. Following overnight incubation in the presence of 20 µg/ml plasminogen, supernatant from these cells was then amplified and plaqued in MDBK cells in the absence of protease in the medium. The appearance of plaques in MDBK cells (Schulman et al., 197, J. Virol. 10: 639-647) indicated the presence of virus which contained the WSN virus NA gene, since supernatant from control experiments of cells infected only with the WSN-HK virus did not produce plaques. In a typical experiment involving the use of a 35 mm dish for the RNP-transfection,  $2.5 \times 10^2$  plaques were observed.

In another control experiment, synthetic NA RNA was used which was derived from plasmid pT3NAv mut 1 (FIG. 16). This RNA differs from the wild type NA RNA derived from pT3NAv by a single nucleotide deletion in the non-translated region of the 5' end (FIG. 16). RNP-transfection of MDBK cells with this RNA and superinfection with WSN-HK virus did not result in the formation of rescued virus. This negative result is readily explained since we have shown in Section 6 and 7, supra, that the essential sequences for the recognition of viral RNA by viral polymerases as well as the packaging signals are located within the 3' and 5' terminal sequences of the viral RNAs. However, we cannot exclude the possibility that rescue of virus using this mutated RNA does occur, albeit at an undetected frequency.

### 8.2.2. RNA Analysis of Rescued Virus

Virus obtained in the rescue experiment was plaque purified, amplified in MDBK cells and RNA was extracted from this preparation. The RNA was then analyzed by electrophoresis on a polyacrylamide gel. FIG. 17 shows the RNA of the helper virus WSN-HK (lane 1) and the synthetic

NA RNA (lane 3), which was transcribed by T3 polymerase from plasmid pT3NAv. The migration pattern of the RNAs of the rescued virus (lane 2) is identical to that of control WSN virus (lane 4). Also, the NA RNAs in lanes 2 and 4 migrate at the same position as the NA RNA derived from cDNA (lane 3) and faster than the HK virus NA band in the helper WSN-HK virus (lane 1). These experiments support the conclusion that as a result of the RNP-transfection, infectious virus was formed containing WSN virus NA RNA derived from cDNA.

### 8.2.3. Rescue of Infectious Influenza Virus Using Virion RNA

In another transfection experiment, RNA extracted from purified WSN virus was employed. When this naked RNA is transfected together with the polymerase proteins into helper virus infected cells, rescue of WSN virus capable of replicating in MDBK cells is observed. RNA isolated from an amplified plaque in this experiment is analyzed in lane 5 of FIG. 17 and shows a pattern indistinguishable from that of the control of WSN virus in lane 4.

### 8.2.4. Introduction of Site-Specific Mutations into the Viral Genome

The experiments described so far involved the rescue of influenza WSN virus. Since the synthetic RNA used in these experiments is identical to the authentic WSN NA gene, the unlikely possibility of contamination by wild type WSN virus could not be rigorously ruled out. Therefore, we introduced five silent point mutations in the coding region of the NA gene in plasmid pT3NAv. These mutations were introduced by cassette mutagenesis through replacement of the short NcoI/PstI fragment present in the NA gene. The five mutations in the cDNA included a C to T change at position 901 and a C to A change at position 925, creating a new XbaI site and destroying the original PstI site, respectively. In addition, the entire serine codon at position 887-889 of the cDNA clone was replaced with an alternate serine triplet (FIG. 17). RNP-transfection of this mutagenized RNA (pT3NAv mut 2) and helper virus infection of MDBK cells again resulted in the rescue of a WSN-like virus which grew in MDBK cells in the absence of added protease. When the RNA of this virus was examined by sequence analysis, all five point mutations present in the plasmid DNA (FIG. 16) were observed in the viral RNA (FIG. 18). Since it is extremely unlikely that these mutations evolved in the wild type influenza WSN virus, we conclude that successful rescue of infectious influenza virus containing five site-specific mutations was achieved via RNP-transfection of engineered RNA.

## 9. RESCUE OF INFECTIOUS RESPIRATORY SYNCYTIAL VIRUSES (RSV) USING RNA DERIVED FROM SPECIFIC RECOMBINANT DNAs.

This example describes a process for the rescue of synthetic RNAs of infectious respiratory syncytial virus (RSV), derived from recombinant cDNAs of first portions of and then the entire RSV RNA genome into stable and infectious RSVs, as noted in Section 5 above. This process can be used in the production of chimeric RSV viruses which can express foreign genes, ie, genes non-native to RSV. Another exemplary way to achieve the production of chimeric RSV involves modifying existing, native RSV genes, as is further described. Accordingly, this example also describes the utility of this process in the directed attenuation of RSV pathogenicity, resulting in production of a vaccine with defined, engineered biological properties for use in humans.

The first step of the rescue process involving portions of the RSV genome requires synthesis of double strand DNA representing the 3' and 5'-terminal extracistronic regions of RSV strain A2 (leader and trailer respectively). The assembly of these extracistronic regions is described in more detail in Section 9.1.2. The bacterial CAT gene, the exemplary heterologous gene coding sequence used in this example, is then flanked with the leader and trailer DNAs to form a miniature RSV 'genome' with the leader region positioned closest to the CAT gene AUG (initiation codon for protein synthesis); see FIG. 21. RNA is transcribed in vitro from this DNA construct and transfected into helper virus-infected (RSV strain A2, or RSV strain B9320) 293 cells. The subsequent presence of CAT activity in these cells indicates the encapsidation, transcription and replication of the RSV/CAT RNA. It will be apparent to one skilled in the art that other heterologous sequences, such as for example, other foreign or non-native genes (i.e. genes from organisms or sources other than RSV), additional RSV genes, native RSV genes or regulatory sequences having specific substitutions, additions and/or deletions in the nucleotide sequence and/or gross alterations in the genetic structure of native RSV, that, in effect, result in heterologous sequences due to their alterations or modifications, can be substituted for the CAT sequence exemplified herein to produce a chimeric RSV.

The first step of the rescue process involving the entire RSV RNA genome requires synthesis of a full length copy of the 15 kilobase (kb) genome of RSV strain A2. This is accomplished by splicing together subgenomic double strand cDNAs (using standard procedures for genetic manipulation) ranging in size from 1 kb–3.5 kb, to form the complete genome cDNA. Determination of the nucleotide sequence of the genome cDNA allows identification of errors introduced during the assembly process; errors can be corrected by site directed mutagenesis, or by substitution of the error region with a piece of chemically synthesized double strand DNA. Following assembly, the genome cDNA is positioned adjacent to a transcriptional promoter (e.g., the T7 promoter) at one end and DNA sequence which allows transcriptional termination at the other end, e.g., a specific endonuclease or a ribozyme, to allow synthesis of a plus or minus sense RNA copy of the complete virus genome in vitro or in cultured cells.

The rescue process utilizes the interaction of full length RSV strain A2 genome RNA, which is transcribed from the constructed cDNA, with helper RSV subgroup B virus proteins inside cultured cells. This can be accomplished in a number of ways. For example, full length virus genomic RNA from RSV strain A2 can be transcribed in vitro and transfected into RSV strain B9320 infected cells, such as 293 cells using standard transfection protocols. In addition, in vitro transcribed genomic RNA from RSV strain A2 can be transfected into a cell line expressing the essential RSV strain A2 proteins (in the absence of helper virus) from stably integrated virus genes.

Alternatively, in vitro transcribed virus genome RNA (RSV strain A2) can also be transfected into cells infected with a heterologous virus (e.g. in particular vaccinia virus) expressing the essential helper RSV strain A2 proteins, specifically the N, P and L proteins. In addition the in vitro transcribed genomic RNA may be transfected into cells infected with a heterologous virus, for example vaccinia virus, expressing T7 polymerase, which enables expression of helper proteins from transfected plasmid DNAs containing the helper N, P and L genes.

As an alternative to transfection of in vitro transcribed genomic RNA, plasmid DNA containing the entire RSV

cDNA construct may be transfected into cells infected with a heterologous virus, for example vaccinia virus, expressing the essential helper RSV strain A2 proteins and T7 polymerase, thereby enabling transcription of the entire RSV genomic RNA from the plasmid DNA containing the RSV cDNA construct. The vaccinia virus need not however, supply the helper proteins themselves but only the T7 polymerase; then helper proteins may be expressed from transfected plasmids containing the RSV N, P and L genes, appropriately positioned adjacent to their own T7 promoters.

Where replicating virus is providing the helper function during rescue experiments, the B9320 strain of RSV is used, allowing differentiation of progeny rescued RSV strain A2 and helper virus RSV strain B9320 with neutralizing monoclonal antibodies directed against RSV B9320. Rescued RSV strain A2 is positively identified by the presence of specific nucleotide 'marker' sequences inserted in the cDNA copy of the RSV genome prior to rescue.

The establishment of a rescue system for native, ie, 'wild-type' RSV strain A2 allows modifications to be introduced into the cDNA copy of the RSV genome to construct chimeric RSV containing sequences heterologous in some manner to that of native RSV, such that the resulting rescued virus may be attenuated in pathogenicity to provide a safe and efficacious human vaccine as discussed in Section 5.4 above. The genetic alterations required to cause virus attenuation may be gross (e.g. translocation of whole genes and/or regulatory sequences within the virus genome), or minor (e.g. single or multiple nucleotide substitution(s), addition(s) and/or deletion(s) in key regulatory or functional domains within the virus genome), as further described in detail.

In addition to alteration(s) (including alteration resulting from translocation) of the RSV genetic material to provide heterologous sequence, this process permits the insertion of 'foreign' genes (i.e., genes non-native to RSV) or genetic components thereof exhibiting biological function or antigenicity in such a way as to give expression of these genetic elements; in this way the modified, chimeric RSV can act as an expression system for other heterologous proteins or genetic elements, such as ribozymes, anti-sense RNA, specific oligoribonucleotides, with prophylactic or therapeutic potential.

## 9.1 Rescue of the Leader and Trailer Sequences of RSV Strain A2 Using RSV Strain B9320 as Helper

### 9.1.1. Viruses and Cells

Although RSV strain A2 and RSV strain B9320 was used in this Example, they are exemplary. It is within the skill in the art to use other strains of RSV subgroup A and RSV subgroup B viruses in accordance with the teachings of this Example. Methods which employ such other strains are encompassed by the invention.

RSV strain A2 and RSV strain B9320 were grown in Hep-2 cells and Vero cells respectively, and 293 cells were used as host during transfection/rescue experiments. All three cell lines were obtained from the ATCC (Rockville, Md.). The RSV strain A2 was also obtained from the ATCC and RSV strain B9320 was a gift from Dr. M. Hendry at the Department of Health Services, Berkeley, Calif.

### 9.1.2. Construction of Plasmids

Plasmid pRSVA2CAT (FIG. 21) was constructed as described below.

The cDNAs of the 44 nucleotide leader and 155 nucleotide trailer components of RSV strain A2 (see Mink et al., *Virology* 185:615–624 (1991); Collins et al., *Proc. Natl.*

*Acad. Sci.* 88:9663–9667 (1991)), the trailer component also including the promoter consensus sequence of bacteriophage T7 polymerase, were separately assembled by controlled annealing of oligonucleotides with partial overlapping complementarity (see FIG. 21). The oligonucleotides used in the annealing were synthesized on an Applied Biosystems DNA synthesizer (Foster City, Calif.). The separate oligonucleotides and their relative positions in the leader and trailer sequences are indicated in FIG. 21. The oligonucleotides used to construct the leader were:

1. 5' CGA CGC ATA TTA CGC GAA AAA ATG CGT ACA ACA  
AAC TTG CAT AAA C
2. 5' CAA AAA AAT GGG GCA AAT AAG AAT TTG ATA AGT  
ACC ACT TAA ATT TAA CT
3. 5' CTA GAG TTA AAT TTA AGT GGT ACT
4. 5' TAT CAA ATT CTT ATT TGC CCC ATT TTT TTG GTT  
TAT GCA AGT TTG TTG TA
5. 5' CGC ATT TTT TCG CGT AAT ATG CGT CGG TAC

The oligonucleotides used to construct the trailer were:

1. 5' GTA TTC AAT TAT AGT TAT TAA AAA TTA AAA ATC ATA  
TAA TTT TTT AAA TA
2. 5' ACT TTT AGT GAA CTA ATC CTA AAG TTA TCA TTT TAA  
TCT TGG AGG AAT AA
3. 5' ATT TAA ACC CTA ATC TAA TTG GTT TAT ATG TGT ATT  
AAC TAA ATT ACG AG
4. 5' ATA TTA GTT TTT GAC ACT TTT TTT CTC GTT ATA GTG  
AGT CGT ATT A
5. 5' AGC TTA ATA CGA CTC ACT ATA ACG A
6. 5' GAA AAA AAG TGT CAA AAA CTA ATA TCT CGT AAT  
TTA GTT AAT ACA CAT AT
7. 5' AAA CCA ATT AGA TTA GGG TTT AAA TTT ATT CCT CCA  
AGA TTA AAA TGA TA
8. 5' ACT TTA GGA TTA GTT CAC TAA AAG TTA TTT AAA AAA  
TTA TAT GAT TTT TA
9. 5' ATT TTT AAT AAC TAT AAT TGA ATA CTG CA

The complete leader and trailer cDNAs were then ligated to the CAT reporter gene XbaI and PstI sites respectively to form a linear ~1 kb RSV/CAT cDNA construct. This cDNA construct was then ligated into the KpnI and HindIII sites of pUC19. The integrity of the final pRSVA2CAT construct was checked by gel analysis of the size of XbaI/PstI and KpnI/HindIII digestion products.

In vitro transcription of HgaI linearized pRSVA2CAT with bacteriophage T7 polymerase was performed according to the T7 supplier protocol (Promega Corporation, Madison, Wis.). Confluent 293 cells in six-well dishes (~1×10<sup>6</sup> cells per well) were infected with RSV strain B9320 at 1 pfu cell<sup>-1</sup> and 1 hour later were transfected with 5–10 µg of the in vitro transcribed RNA from the pRSVA2CAT construct. The transfection procedure followed the transfection procedure of Collins et al., *Virology* 195:252–256 (1993) and

employed Transfect/ACE™ and Optimem reagents according to the manufacturers specifications (Gibco-BRL, Bethesda, Md.). At 24 hours post infection the 293 cells were assayed for CAT activity using a standard protocol (Current Protocols in Molecular Biology, Vol. 1, Chapter 9.6.2; Gorman, et al., (1982) *Mol. Cell. Biol.* 2:1044–1051). The detection of high levels of CAT activity indicated that in vitro transcribed negative sense RNA containing the 'leader' and 'trailer' regions of the RSV A2 strain genome and the CAT gene can be encapsidated, replicated and expressed using proteins supplied by RSV strain B9320 (See FIG. 22). The level of CAT activity observed in these experiments was at least as high as that observed in similar rescue experiments where homologous RSV strain A2 was used as helper virus. This ability of an antigenically distinct subgroup B RSV strain B9320 to support the encapsidation, replication and transcription of a subgroup A RSV strain A2 RNA has to our knowledge hitherto not been formally reported. In view of the fact that influenza B virus is unable to provide analogous helper activity for similar CAT constructs containing influenza A leader and trailer sequences, these observations are therefore surprising and unexpected.

## 9.2 Construction and Rescue of a cDNA Representing the Complete Genome of RSV

To obtain a template for cDNA synthesis, RSV genomic RNA, comprising 15,222 nucleotides, is purified from infected Hep-2 cells according to the method described by Ward et al., *J. Gen. Virol.* 64:1867–1876 (1983). Based on the published nucleotide sequence of RSV, oligonucleotides are synthesized using an Applied Biosystems DNA synthesizer (Applied Biosystems, Foster City, Calif.) to act as primers for first and second strand cDNA synthesis from the genomic RNA template. The nucleotide sequences and the relative positions of the cDNA primers and key endonuclease sites within the RSV genome are indicated in FIG. 23. The production of cDNAs from virus genomic RNA is carried out according to the reverse transcription/polymerase chain reaction (RT/PCR) protocol of Perkin Elmer Corporation, Norwalk, Conn. (see also Wang et al., (1989) *Proc. Natl. Acad. Sci.* 86:9717–9721); the amplified cDNAs are purified by electro elution of the appropriate DNA band from agarose gels. Purified DNA is ligated directly into the pCRII plasmid vector (Invitrogen Corp. San Diego), and transformed into either 'One Shot' *E. coli* cells (Invitrogen) or 'SURE' *E. coli* cells (Stratagene, San Diego). The resulting, cloned, virus specific, cDNAs are assembled by standard cloning techniques (Sambrook et al., *Molecular Cloning—A Laboratory manual*, Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y., 1989) to produce a cDNA spanning the complete RSV genome. The entire cDNA genome is sequenced, and incorrect sequence replaced by either site-directed mutagenesis or chemically synthesized DNA. Nucleotide substitutions are introduced at bases 7291 and 7294 (with base number 1 being at the start of the genomic RNA 3' end) in the 'F' gene, to produce a novel StuI endonuclease site, and at positions 7423, 7424, and 7425 (also in the F gene) to produce a novel PmeI site. These changes are designed to act as definitive markers for rescue events. The bacteriophage T7 polymerase and the HgaI endonuclease site are placed at opposite ends of the virus genome cDNA such that either negative or positive sense virus genome RNA can be synthesized in vitro. The cDNAs representing the T7 polymerase promoter sequence and the recognition sequence for HgaI were synthesized on an Applied Biosystems DNA synthesizer and are separately ligated to the ends of the virus genome cDNA, or are added

as an integral part of PCR primers during amplification of the terminal portion of the genome cDNA, where appropriate; the latter procedure is used when suitable endonuclease sites near the genome cDNA termini are absent, preventing direct ligation of chemically synthesized T7 promoter/Hgal site cDNA to the genome cDNA. This complete construct (genome cDNA and flanking T7 promoter/Hgal recognition sequence) is then cloned into the KpnI/NotI sites of the Bluescript II SK phagemid (Stratagene, San Diego) from which the endogenous T7 promoter has been removed by site-directed mutagenesis. RNA transcribed from this complete genome construct may be rescued using RSV subgroup B helper virus to give infectious RSV in accordance with Example 9.1. This basic rescue system for the complete native, i.e., 'wild-type' RSV A2 strain genomic RNA can be employed to introduce a variety of modifications into the cDNA copy of the genome resulting in the introduction of heterologous sequences into the genome. Such changes can be designed to reduce viral pathogenicity without restricting virus replication to a point where rescue becomes impossible or where virus gene expression is insufficient to stimulate adequate immunity.

These modifications can comprise gross alterations of the genetic structure of RSV, such as gene shuffling. For example, the RSV 'M<sub>1</sub>' gene can be translocated to a position closer to the 5' end of the genome, in order to take advantage of the known 3' to 5' gradient in virus gene expression, resulting in reduced levels of 'M<sub>1</sub>' protein expression in infected cells and thereby reducing the rate of virus assembly and maturation. Other genes and/or regulatory regions may also be translocated appropriately, in some cases from other strains of RSV of human or animal origin. For example, the 'F' gene (and possibly the 'G' gene) of a human subgroup B RSV could be inserted into an otherwise RSV strain A genome (in place of, or in addition to the RSV strain A 'F' and 'G' genes).

In another approach, the RNA sequence of the RSV viruses 'N' protein is translocated from its 3' proximal site to a position closer to the 5' end of the genome, again taking advantage of the 3' to 5' gradient in gene transcription to reduce the level of N protein produced. By reducing the level of N protein produced, there would result a concomitant increase in the relative rates of transcription of genes involved in stimulating host immunity to RSV and a concomitant reduction in the relative rate of genome replication. Thus, by translocating the RSV RNA sequence coding for RSV N protein, a chimeric RSV virus having attenuated pathogenicity relative to native RSV will be produced.

Another exemplary translocation modification resulting in, in effect, the introduction of heterologous sequence as is discussed herein may result in the production of attenuated chimeric RSV comprises the translocation of RSV RNA sequence coding for the 'L' protein of RSV. This sequence of the RSV virus is believed responsible for viral polymerase protein production. By translocating the RSV sequence coding for L protein from its native 5' terminal location in the native RSV genome to a location at or near the 3' terminus of the genome, a chimeric RSV virus exhibiting attenuated pathogenicity will be produced. Yet another exemplary translocation comprises the switching the locations of the RSV RNA sequences coding for the RSV G and F proteins (ie, relative to each other in the genome) to achieve a chimeric RSV having attenuated pathogenicity resulting from the slight modification in the amount of the G and F proteins produced. Such gene shuffling modifications as are exemplified and discussed above are believed to result in a chimeric, modified RSV having attenuated pathogenic-

ity in comparison to the native RSV starting material. The nucleotide sequences for the foregoing encoded proteins are known, as is the nucleotide sequence for the entire RSV genome. See McIntosh, Respiratory Syncytial Virus in Virology, 2d Ed. edited by B. N. Fields, D. M. Knipe et al., Raven Press, Ltd. New York, 1990 Chapter 38, pp1045-1073, and references cited therein.

These modifications can additionally or alternatively comprise localized, site specific, single or multiple, nucleotide substitutions, deletions or additions within genes and/or regulatory domains of the RSV genome. Such site specific, single or multiple, substitutions, deletions or additions can reduce virus pathogenicity without overly attenuating it, for example, by reducing the number of lysine or arginine residues at the cleavage site in the F protein to reduce efficiency of its cleavage by host cell proteases (where cleavage is believed to be an essential step in functional activation of the 'F' protein), and thereby possibly reduce virulence. Site specific modifications in the 3' or 5' regulatory regions of the RSV genome may also be used to increase transcription at the expense of genome replication. In addition, localized manipulation of domains within the N protein, which is believed to control the switch between transcription and replication can be made to reduce genome replication but still allow high levels of transcription. Further, the cytoplasmic domain(s) of the G and F glycoproteins can be altered in order to reduce their rate of migration through the endoplasmic reticulum and golgi of infected cells, thereby slowing virus maturation. In such cases, it may be sufficient to modify the migration of G protein only, which would then allow additional up-regulation of 'F' production, the main antigen involved in stimulating neutralizing antibody production during RSV infections. Such localized substitutions, deletions or additions within genes and/or regulatory domains of the RSV genome are believed to result in chimeric, modified RSV also having reduced pathogenicity relative to the native RSV genome starting material.

### 9.3 Use of Monoclonal Antibodies to Differentiate Rescued Virus from Helper Virus

In order to neutralize the RSV strain B9320 helper virus and facilitate identification of rescued A2 strain RSV, monoclonal antibodies against RSV strain B9320 were made as follows.

Six BALB/c female mice were infected intranasally (i.n.) with  $\sim 10^5$  plaque forming units (p.f.u.) of RSV B9320, followed 5 weeks later by intraperitoneal (i.p.) inoculation with  $10^6$ – $10^7$  pfu of RSV B9320 in a mixture containing 50% complete Freund's adjuvant. Two weeks after i.p. inoculation, a blood sample from each mouse was tested for the presence of RSV specific antibody using a standard neutralization assay (Beeler and Coelingh, *J. Virol.* 63:2941-2950 (1988)). Mice producing the highest level of neutralizing antibody were then further boosted with  $\sim 10^6$  p.f.u. of RSV strain B9320 in phosphate buffered saline (PBS), injected intravenously at the base of the tail. Three days later, the mice were sacrificed and their spleens collected as a source of monoclonal antibody producing B-cells. Spleenocytes (including B-cells) were teased from the mouse spleen through incisions made in the spleen capsule into 5 ml of Dulbecco's Modified Eagle's Medium (DME). Clumps of cells were allowed to settle out, and the remaining suspended cells were separately collected by centrifugation at  $\sim 2000 \times g$  for 5 minutes at room temperature. These cell pellets were resuspended in 15 ml 0.83% (w/v)  $\text{NH}_4\text{Cl}$ , and allowed to stand for 5 minutes to lyse red

blood cells. Spleenocytes were then collected by centrifugation as before through a 10 ml cushion of fetal calf serum. The spleenocytes were then rinsed in DME, repelleted and finally resuspended in 20 ml of fresh DME. These spleenocytes were then mixed with Sp2/0 cells (a mouse myeloma cell line used as fusion partners for the immortalization of spleenocytes) in a ratio of ~10:1, spleen cells: Sp2/0 cells. Sp2/0 cells were obtained from the ATCC and maintained in DME supplemented with 10% fetal bovine serum. The cell mixture was then centrifuged for 8 minutes at 2000xg at room temperature. The cell pellet was resuspended in 1 ml of 50% polyethylene glycol 1000 mol. wt. (PEG 1000), followed by addition of equal volumes of DME at 1 minute intervals until a final volume of 25 ml was attained. The fused cells were then pelleted as before and resuspended at  $3.5 \times 10^6$  spleen cells  $\text{ml}^{-1}$  in growth medium (50% conditioned medium from Sp2/0 cells, 50% HAT medium containing 100 ml RPMI 25 ml F.C.S.,  $100 \mu\text{gml}^{-1}$  gentamicin, 4 ml 50x Hypoxanthine, Thymidine, Aminopterin (HAT) medium supplied as a prepared mixture by Sigma Chem. Co., St. Louis, Mo.). The cell suspension was distributed over 96 well plates ( $200 \mu\text{l}$  well $^{-1}$ ) and incubated at 37° C., 95% humidity and 5% CO<sub>2</sub>. Colonies of hybridoma cells (fused spleenocytes and Sp2/0 cells) were then subcultured into 24 well plates and grown until nearly confluent; the supernatant growth medium was then sampled for the presence of RSV strain B9320 neutralizing monoclonal antibody, using a standard neutralization assay (Beeler and Coelingh, *J. Virol.* 63:2941-50 (1988)). Hybridoma cells from wells with neutralizing activity were resuspended in growth medium and diluted to give a cell density of ~0.5 cells per  $100 \mu\text{l}$  and plated out in 96 well plates,  $200 \mu\text{l}$  per well. This procedure ensured the production of monoclones (i.e. hybridoma cell lines derived from a single cell) which were then reassayed for the production of neutralizing monoclonal antibody. Those hybridoma cell lines which produced monoclonal antibody capable of neutralizing RSV strain B9320 but not RSV strain A2 were subsequently injected into mice, i.p. ( $10^6$  cells per mouse). Two weeks after the i.p. injection mouse ascites fluid containing neutralizing monoclonal antibody for RSV strain B9320 was tapped with a 19 gauge needle, and stored at -20° C.

This monoclonal antibody was used to neutralize the RSV strain B9320 helper virus following rescue of RSV strain A2 as described in Section 9.1. This was carried out by diluting neutralizing monoclonal antibody 1 in 50 with molten 0.4% (w/v) agar in Eagle's Minimal Essential Medium (EMEM)

containing 1% F.C.S. This mixture was then added to Hep-2 cell monolayers, which had been infected with the progeny of rescue experiments at an m.o.i. of ~0.1-0.01 pfu cell $^{-1}$ . The monoclonal antibody in the agar overlay inhibited the growth of RSV strain B9320, but allowed the growth of RSV strain A2, resulting in plaque formation by the A2 strain. These plaques were picked using a pasteur pipette to remove a plug of agar above the plaque and the infected cells within the plaque; the cells and agar plug were resuspended in 2 ml of EMEM, 1 % FCS, and released virus was plaqued again in the presence of monoclonal antibody on a fresh Hep-2 cell monolayer to further purify from helper virus. The twice plaqued virus was then used to infect Hep-2 cells in 24 well plates, and the progeny from that were used to infect 6 well plates at an m.o.i. of ~0.1 pfu cell $^{-1}$ . Finally, total infected cell RNA from one well of a 6 well plate was used in an RT/PCR reaction using first and second strand primers on either side of the 'marker sequences' (introduced into the RSV strain A2 genome to act as a means of recognizing rescue events) as described in Section 9.2 above. The DNA produced from the RT/PCR reaction was subsequently digested with *Stu*I and *Pme*I to positively identify the 'marker sequences' introduced into RSV strain A2 cDNA, and hence to establish the validity of the rescue process.

## 10. DEPOSIT OF MICROORGANISMS

An *E. coli* cell line containing the plasmid pIVACAT is being deposited with the Agricultural Research Culture Collection (NRRL), Peoria, IL; and has the following accession number

Strain	Plasmid	Ascension Number
<i>E. coli</i> (DH5a)	pIVACAT	NRRL

The present investigation is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and any constructs, viruses or enzymes which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims. 78.

## SEQUENCE LISTING

### ( 1 ) GENERAL INFORMATION:

( i i i ) NUMBER OF SEQUENCES: 43

### ( 2 ) INFORMATION FOR SEQ ID NO:1:

#### ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 95 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( i x ) SEQUENCE DESCRIPTION: SEQ ID NO:1:

-continued

---

GAAGCTTAAT ACGACTCACT ATAAGTAGAA ACAAGGGTGT TTTTTCATAT CATTTAAACT	6 0
TCACCCTGCT TTTGCTGAAT TCATTCTTCT GCAGG	9 5

## ( 2 ) INFORMATION FOR SEQ ID NO:2:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 15 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: SYNTHETIC DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCTGCAGAAG AATGA	1 5
------------------	-----

## ( 2 ) INFORMATION FOR SEQ ID NO:3:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 95 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: SYNTHETIC DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAAGCTTAAT ACGACTCACT ATAAGCAAAA GCAGGGTGAA GTTTAAATGA TATGAAAAAA	6 0
CACCCCTTGTT TCTACTGAAT TCATTCTTCT GCAGG	9 5

## ( 2 ) INFORMATION FOR SEQ ID NO:4:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 68 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: SYNTHETIC DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGCTTAATAC GACTCACTAT AAGATCTATT AAACCTCACC CTGCTTTTGC TGAATTCATT	6 0
CTTCTGCA	6 8

## ( 2 ) INFORMATION FOR SEQ ID NO:5:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 60 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: SYNTHETIC DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAAGAATGAA TTCAGCAAAA GCAGGGTGAA GTTTAATAGA TCTTATAGTG AGTCGTATTA	6 0
---	-----

## ( 2 ) INFORMATION FOR SEQ ID NO:6:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 42 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: SYNTHETIC DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:6:

-continued

CCGAATTCTT AATACGACTC ACTATAAGTA GAAACAAGGG TG

4 2

( 2 ) INFORMATION FOR SEQ ID NO:7:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 30 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCTCTAGACG CTCGAGAGCA AAAGCAGGTG

3 0

( 2 ) INFORMATION FOR SEQ ID NO:8:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 15 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CACCCUGCUU UUGCU

1 5

( 2 ) INFORMATION FOR SEQ ID NO:9:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 15 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC RNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CACCCUGCUU UUA CU

1 5

( 2 ) INFORMATION FOR SEQ ID NO:10:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 15 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC RNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CACCCUGCUU CUGCU

1 5

( 2 ) INFORMATION FOR SEQ ID NO:11:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 15 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC RNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CACCCUGUUU UUGCU

1 5

( 2 ) INFORMATION FOR SEQ ID NO:12:

-continued

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 15 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: SYNTHETIC RNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:12:

C A C C C U G U U U U U A C U

1 5

## ( 2 ) INFORMATION FOR SEQ ID NO:13:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 15 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: SYNTHETIC RNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:13:

C A C C C U G U U U U C U G C U

1 5

## ( 2 ) INFORMATION FOR SEQ ID NO:14:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 16 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: SYNTHETIC RNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:14:

C A C C C U U G C U U U U G C U

1 6

## ( 2 ) INFORMATION FOR SEQ ID NO:15:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 16 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: SYNTHETIC RNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:15:

C A C C C U U G C U U U U A C U

1 6

## ( 2 ) INFORMATION FOR SEQ ID NO:16:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 16 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: SYNTHETIC RNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:16:

C A C C C U U G U U U U U A C U

1 6

## ( 2 ) INFORMATION FOR SEQ ID NO:17:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 16 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single



-continued

( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC RNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CACCCUUGUU UCUACU

16

( 2 ) INFORMATION FOR SEQ ID NO:18:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 96 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: double
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTAGACGCCC TGCAGCAAAA GCAGGGTGAC AAAGACATAA TGGAGAAAAA AATCACTGGG

60

TATACCACCG TTGATATATC CCAATCGCAT CGTAAA

96

( 2 ) INFORMATION FOR SEQ ID NO:19:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 96 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: double
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGCGGGACGT CGTTTTCGTC CCACTGTTTC TGTATTACCT CTTTTTTTAG TGACCCATAT

60

GGTGGCAACT ATATAGGGTT AGCGTAGCAT TTCTTG

96

( 2 ) INFORMATION FOR SEQ ID NO:20:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 34 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:20:

actgcgatga gtggcagggc ggggcgtaat agat

34

( 2 ) INFORMATION FOR SEQ ID NO:21:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 38 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:21:

tgacgctact caccgtcccg cccgcatta tctagatc

38

( 2 ) INFORMATION FOR SEQ ID NO:22:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 34 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

-continued

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ACTGCGATGA GTGGCAGGGC GGGGCGTAAT AGAT

3 4

( 2 ) INFORMATION FOR SEQ ID NO:23:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 38 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTAGATCTAT TACGCCCCGC CCTGCCACTC ATCGCAGT

3 8

( 2 ) INFORMATION FOR SEQ ID NO:24:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 97 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTAGACGCCC TGCAGCAAAA GCAGGGTGAC AAAGACATAA TGGAGAAAAA AAATCACTGG

6 0

GTATACCACC GTTGATATAT CCAATCGCA TCGTAAA

9 7

( 2 ) INFORMATION FOR SEQ ID NO:25:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 38 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GTTCTTTACG ATGCGATTGG GATTTTGCTG CAGGGCGT

3 8

( 2 ) INFORMATION FOR SEQ ID NO:26:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 30 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CGGAATTCTC TTCGAGCGAA AGCAGGAGTT

3 0

( 2 ) INFORMATION FOR SEQ ID NO:27:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 42 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

-continued

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCAAGCTTAT TAACCCTCAC TAAAAGTAGA AACAAAGGAGT TT

4 2

( 2 ) INFORMATION FOR SEQ ID NO:28:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 51 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CATGGGTGAG TTTCGACCAA AATCTAGATT ATAAAATAGG ATACATATGC A

5 1

( 2 ) INFORMATION FOR SEQ ID NO:29:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 43 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AATGTATCCT ATTTTATAAT CTAGATTTTG GTCGAAACTC ACC

4 3

( 2 ) INFORMATION FOR SEQ ID NO:30:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 46 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CGACGCATAT TACGCGAAAA AATGCGTACA ACAAACCTTGC ATAAAC

4 6

( 2 ) INFORMATION FOR SEQ ID NO:31:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 50 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CAAAAAAATG GGGCAAATAA GAATTTGATA AGTACCACTT AAATTTAACT

5 0

( 2 ) INFORMATION FOR SEQ ID NO:32:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 24 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CTAGAGTTAA ATTTAAGTGG TACT

2 4

-continued

## ( 2 ) INFORMATION FOR SEQ ID NO:33:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 50 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: SYNTHETIC DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TATCAAATTC TTATTTGCC CATT TTTT TTTG GTTTATGCAA GTTTGTTGTA

5 0

## ( 2 ) INFORMATION FOR SEQ ID NO:34:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 30 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: SYNTHETIC DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CGCATTTTT CCGCTAATAT GCGTCGGTAC

3 0

## ( 2 ) INFORMATION FOR SEQ ID NO:35:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 50 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: SYNTHETIC DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GTATTCAATT ATAGTTATTA AAAATTAAAA ATCATATAAT TTTTAAATA

5 0

## ( 2 ) INFORMATION FOR SEQ ID NO:36:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 50 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: SYNTHETIC DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:36:

ACTTTTAGTG AACTAATCCT AAAGTTATCA TTTTAATCTT GGAGGAATAA

5 0

## ( 2 ) INFORMATION FOR SEQ ID NO:37:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 50 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: SYNTHETIC DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:37:

ATTTAAACCC TAATCTAATT GGTTTATATG TGTATTAAC TAAATTACGAG

5 0

## ( 2 ) INFORMATION FOR SEQ ID NO:38:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 46 bases
- ( B ) TYPE: nucleic acid

-continued

- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:38:

ATATTAGTTT TTGACACTTT TTTTCTCGTT ATAGTGAGTC GTATTA

4 6

( 2 ) INFORMATION FOR SEQ ID NO:39:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 25 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:39:

AGCTTAATAC GACTCACTAT AACGA

2 5

( 2 ) INFORMATION FOR SEQ ID NO:40:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 50 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GAAAAAAAGT GTCAAAACT AATATCTCGT AATTTAGTTA ATACACATAT

5 0

( 2 ) INFORMATION FOR SEQ ID NO:41:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 50 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:41:

AAACCAATTA GATTAGGGTT TAAATTTATT CCTCCAAGAT TAAAATGATA

5 0

( 2 ) INFORMATION FOR SEQ ID NO:42:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 50 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:42:

ACTTTAGGAT TAGTTCCTA AAAGTTATTT AAAAAATTAT ATGATTTTAA

5 0

( 2 ) INFORMATION FOR SEQ ID NO:43:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 29 bases
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: SYNTHETIC DNA

-continued

( \* i ) SEQUENCE DESCRIPTION: SEQ ID NO:43:

ATTTTAAATA ACTATAATTG AATACTGCA

29

What is claimed is:

1. A recombinant RNA molecule comprising a binding site specific for an RNA-directed RNA polymerase derived from a respiratory syncytial virus, operatively linked to a heterologous RNA sequence comprising the reverse complement of an mRNA coding sequence.

2. A recombinant RNA molecule comprising a heterologous RNA sequence comprising the reverse complement of an mRNA coding sequence, operatively linked to a 3'-noncoding viral sense flanking sequence of a respiratory syncytial virus containing the viral polymerase binding site, and to a 540 -noncoding viral sense flanking sequence of respiratory syncytial virus.

3. A recombinant RNP comprising the recombinant RNA molecule of claim 1 mixed with the purified RNA-directed RNA polymerase.

4. A recombinant RNP comprising the recombinant RNA molecule of claim 3 mixed with purified respiratory syncytial viral polymerase.

5. A recombinant RNP comprising the recombinant RNA molecule of claim 2 mixed with purified respiratory syncytial viral polymerase.

6. A chimeric virus comprising a respiratory syncytial virus containing a heterologous RNA sequence comprising the reverse complement of an mRNA coding sequence, operatively linked to a respiratory syncytial viral polymerase binding site.

7. A chimeric virus comprising a respiratory syncytial virus containing a heterologous RNA sequence comprising the reverse complement of an mRNA coding sequence, operatively linked to a polymerase binding site of the respiratory syncytial virus.

8. A method for producing a chimeric respiratory syncytial virus (RSV) comprising:

(a) culturing a host cell transfected with a heterologous RNA sequence comprising the reverse complement of an mRNA coding sequence operatively linked to a RSV polymerase binding site and infected with a parental strain of RSV and,

(b) recovering said chimeric virus from the culture.

9. A chimeric RSV produced by the method of claim 8.

\* \* \* \* \*

BOOK 1

LABORATORY  
NOTEBOOK

DAVID K. CLARKE

ATLANTA

0000001

Reference binder #22A



**NOTEBOOK NO.** 1  
**ISSUED TO** DAVID K. CLARKE  
**ON** \_\_\_\_\_  
**DEPARTMENT** AVIRON.  
**RETURNED** \_\_\_\_\_

—SCIENTIFIC NOTEBOOK CO.—  
2831 LAWRENCE AVE.  
P.O. BOX 238  
STEVENSVILLE, MI 49127  
616-429-8285

0000003

## INSTRUCTIONS

1. **The primary purpose of this notebook is to protect your and the Company's Patent-Rights by keeping records of all original work in a form acceptable as evidence if any legal conflict arises.**
2.
  - When starting a page, enter the title, project number, and book number.
  - Use ink for permanence -- avoid pencil.
  - Record your work as you progress, including any spur-of-the-moment ideas which may be developed later.
  - Avoid making notes on loose paper to be recopied.
  - Record your work in such a manner that a co-worker can continue from where you stop. You might be ill and to protect your priority it could be urgent that the work continue while you are absent.
3.
  - Give a complete account of your experiments and the results, both positive and negative, including your observations.
  - Record all diagrams, layouts, plans, procedures, new ideas, or anything pertinent to your work including the details of any discussions with suppliers, or other people outside the Company.
  - Do not try to erase any incorrect entries; draw lines deleting them, note the corrections, sign and date the changes. This extra care is worthwhile because of the necessity of original data to prove priority of new discoveries.
4.
  - After entering your data, sign and date the entries.
  - Explain your work to at least two witnesses who are not co-inventors, and have them sign and date the pages in the place provided.
  - Record the names of operators and witnesses present during any demonstration and have at least two witnesses sign the page. If no witnesses are present during an experiment of importance, repeat it in the presence of two witnesses.
5. Since computer programs can be patented these instructions apply to the development of computer software. In this case a description of the structure and operation of the program should be recorded in the notebook, together with a basic flow diagram which illustrates the essential features of the program. In the course of developing the code, the number of lines of code written each day should be recorded in the notebook, together with a statement of the portion of the flow diagram to which the section of code is directed.
6. This notebook and its contents are the exclusive property of the Company. It is confidential and the contents are not to be disclosed to anyone unless authorized by the Company. You must return it when completed, upon request, or upon termination of employment. It should be kept in a protected place. **If loss occurs, notify your supervisor immediately, and make a written report describing the circumstances of the loss.**





































293 cells

**.HEADLINE**

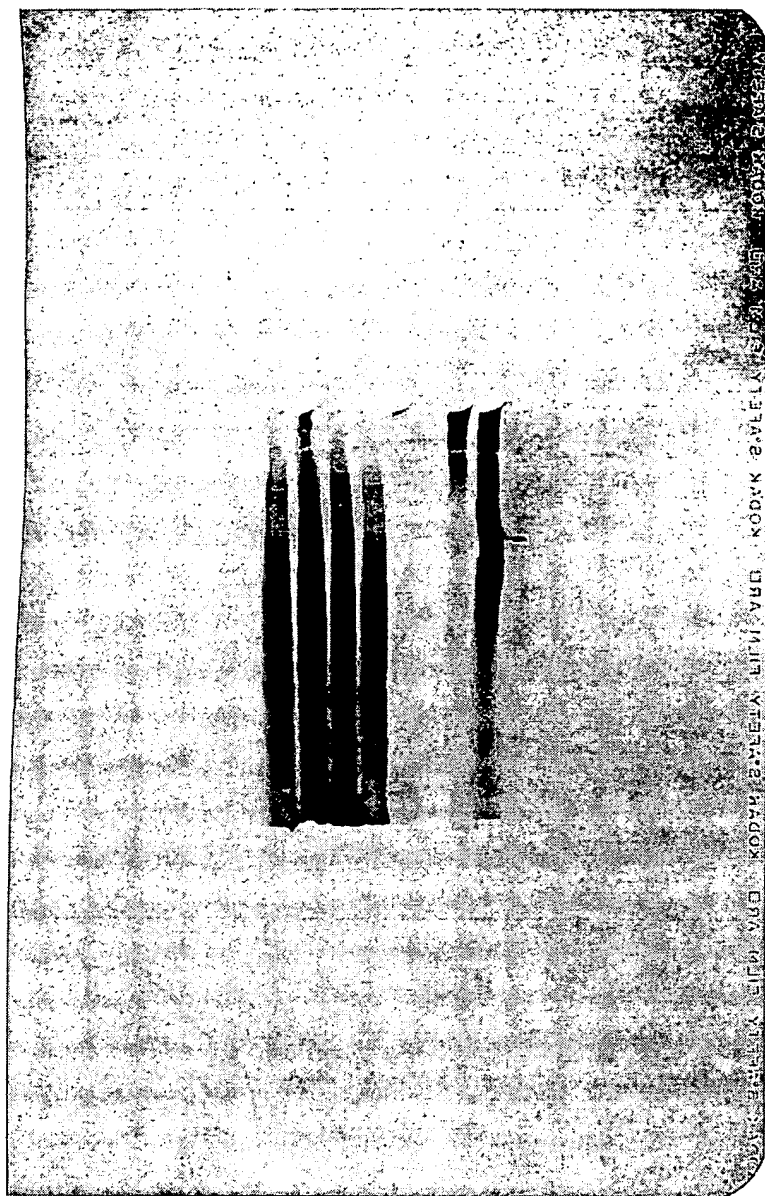
ATCC CRL-1573 293 (Transformed primary embryonal kidney, human)

**.TEXT**

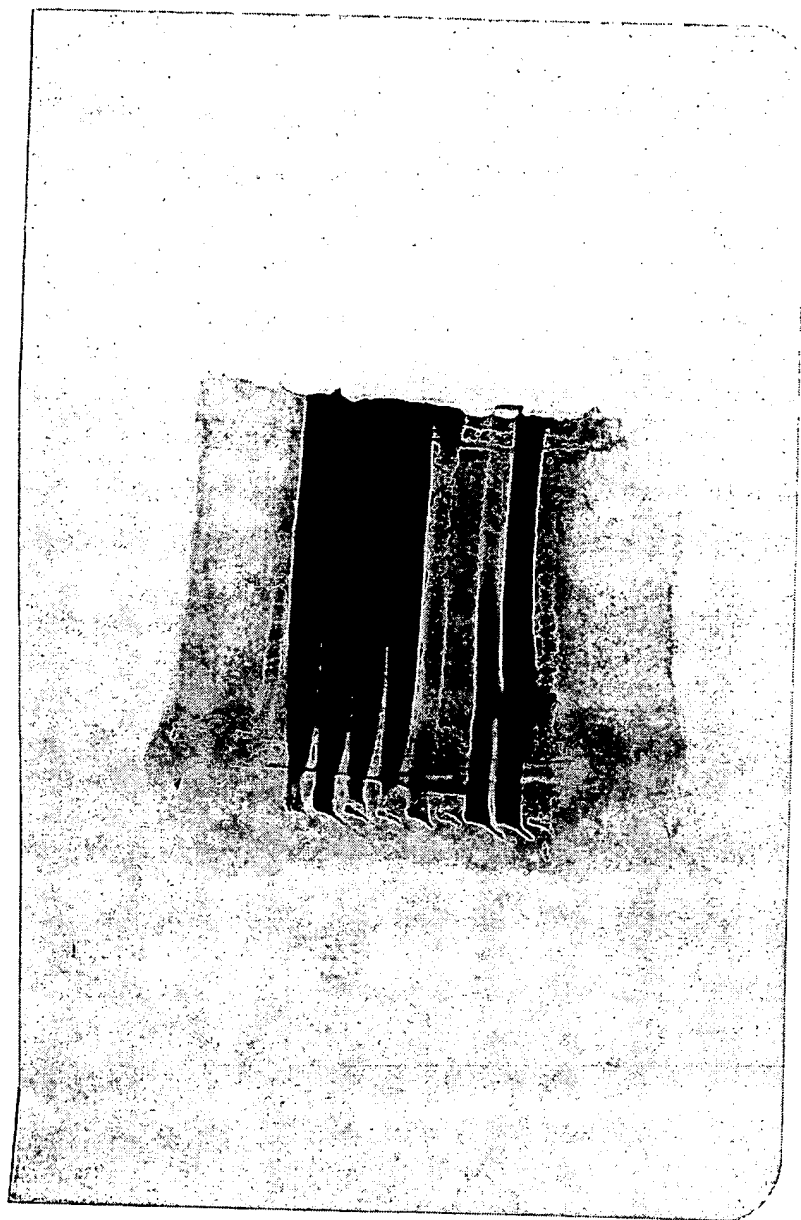
Passage Frozen: 31. Current medium for propagation: Eagle's MEM with Earle's BSS, 90%; heat-inactivated horse serum, 10%. Additional Information: The 293 cell line is a permanent line of primary human embryonal kidney transformed by sheared human adenovirus type 5 (Ad 5) DNA. The cells are particularly sensitive to human adenovirus, are highly permissive for adenovirus DNA, and contain and express the transforming genes of Ad 5. Handle as potentially biohazardous material under at least Biosafety Level 2 containment. The line has been used in the isolation of transformation defective, host-range mutants of Ad 5, and is excellent for titrating human adenoviruses. This is a hypotriploid human cell line. The modal chromosome number was 64, occurring in 30% of cells. The rate of cells with higher ploidies was 4.2 %. The der(1)t(1;15) (q42;q13), der(19)t(3;19) (q12;q13), der(12)t(8;12) (q22;p13), and four other marker chromosomes were common to most cells. Five other markers occurred in some cells only. The marker der(1) and M8 (or Xq+) were often paired. There were four copies of N17 and N22. Noticeably in addition to three copies of X chromosomes, there were paired Xq+, and a single Xp+ in most cells. References: J. Gen. Virol. 36: 59-72, 1977; Virology 77: 319-329, 1977; *ibid.*, 86: 10-21, 1978. Submitted by: F.L. Graham, McMaster University, Hamilton, Ontario, Canada. Price Code: J

*Age W-Kha*

000021



000022



000023

From Page No. \_\_\_\_\_

1<sup>st</sup> Week at Aviron was spent meeting people, finding out how the various support systems operated; getting a desk/office organised, and reading background material on RSV, and Herpes Viruses.

Looked over protocol for RSV growth and storage. Identified RSV subgroups (A) and (B) in Kathy's freezer box.

Prepared EMEM supplemented with

(A) = pp 3D AZ HEp4

(B) = pp 3 18537 HEp4

(1) Glutamine (1% v/v - stock  $29.3g L^{-1}$ )

(2) Streptomycin } 1% v/v - stock  $25g L^{-1}$   
Penicillin G } - stock  $15,779g L^{-1}$

(3) FCS 1% heat inactivated.

Infected  $2 \times 75cm^2$  monolayers of Vero cells, each with  $200\mu l$  ( $\approx 10^6$  pfu/ml) of RSV. Used 2 Flasks for subtype A and 2 Flasks for subtype B.

Adsorbed virus on monolayer for 1-2 hrs - then added EMEM ( $10ml$  ~~100ml~~) and continued incubation to observe C.P.E.

Prepared separate stocks of 1M Hepes and 2M MgSO<sub>4</sub> ( $100ml$ ) Autoclaved and stored in tissue culture room.

Checked cytopathic effect of (AZ) and (18537) on Vero cell. (under phase contrast) There appears to be some cytopathic effect, apparent at localised regions of the monolayers. The main effect appears as a fusion of cell membranes to form syncytia.

To Page No. 2

Witnessed &amp; Understood by me,

*Holly Abrams*

Date

Invented by

Date

Recorded by

*David K Clarke*

000024

From Page No. 1

Need to order in the future.

- ① Phenol chloroform\*
- ② Methyl Mercury\*
- ③ Tag. Polymerase
- ④ Reverse Transcriptase
- ⑤ Mechanical stirrer\* ✓
- ⑥ HEP-2 cells\* ✓

\* Immediately

Reverse Transcriptase should be Avian from Life Sciences St. Petersburg, Florida

1-800-237-4323

CATALOG NO. AZM-007

1000 u/vial.

Prepared stock solutions of 20% SDS (ultra pure)

Prepared a stock solution of 1M  $MgCl_2$ Autoclaved along with a Deionized  $H_2O$  supply.

am. Checked cytopathic effect on Vero cells again. Noted extensive C.P.E. with AZ in both Flasks. The C.P.E. was evident as large areas of fused cells which also looked v. sickly and 'grainy'. Vero infected with sub-type B: showed only v. limited and localized C.P.E. with small areas of fusion - will check both again later today.

Ordering info:

Magnetic Stirrer

E and K Scientific Products, Inc.

CAT NO. 500P Price \$185

Received

AVIAN REV. TRANSCRIPTASE

Life Sciences Inc.

2900 72<sup>ND</sup> STREET NORTH

St. Petersburg

1000 u - \$125 +

Florida 33710

Fax (813) 347-2957

#35 Freight

SATURATED PHENOL/CHLOROFORM

AMRESCO

Product Code 0945

100ML \$25-80

arrives ON an Dry Ice.

No shipping on Frig.

To Page No. 2

Witnessed &amp; Understood by me,

Date

Invented by

Date

Holly Abrams

recorded by

DAVID K. CLARKE

000025

**'TITLE'**

From Page No. 2

### Proposed scheme for utilisation of virus stocks.

A2 and 18537 (1 ml vials from NIH)

$\frac{1}{2}$  ml / Flask  $75 \text{ cm}^2$   $\frac{1}{2}$  ml / Flask.

20ml

Harvested and  
used as stock

20ml

Harvested and used as stock. (Label carefully)

- Try new cell lines.

- In different M.O.I.  $\Rightarrow$  m

- Try different incubat<sup>n</sup> temp.

Preparation of working stocks.  
(200-400ml)

## Preparation of working stocks (200-400 ml)

ATCC ACC. N°  
11735

~~2 100 Roller bottles~~  
~~Worth~~

4

Ordered. HEp-2 cells 2 x 1 ml vials at x \$97-w vial. (ATCC)

4-30pm initiated harvest of RSV A2 due to advanced c.p.e. Followed protocol

- 1) Scraped infected cells into supernatant
- 2) centrifuged urine at  $4^{\circ}\text{C}$  3,000 rpm.
- 3) Decanted super into clean tube - resuspended pellet in 2ml fresh EMEM, and went through freeze-thaw cycle - repelleted and poured super with 1<sup>st</sup> - Added 1ml 1M Hepes, 1ml of 2M  $\text{MgSO}_4$  (added v. alkaline pH - adjusted with conc. HCl) Aliquotted virus to 1ml vials - froze in dry-ethanol ice bath. Then stored virus at  $-70^{\circ}\text{C}$



From Page No. 3.

Checked c.p.e. on 18537 infected cells — there were numerous localised areas of multicellular syncytial formation, but not sufficiently widespread to warrant harvesting. Poured off old medium and added 10ml fresh medium to each flask, in an attempt to stimulate virus production — will check again this afternoon and tomorrow morning.

Local supplier of Methyl mercury II hydroxide

Received  
Stored in  
Fume hood  
Dues/Well 14

Alpha-Aesar. — Fax 1-800-322-4757 Tel. 1-800-343-1990

Fed ID No. Required

Book Ref.

CAT. No. 13395 50ml = \$295

Ordered Phenol chloroform from Amresco and Methyl mercury from Aesar

Received  
6/20/90

Set up TCID<sub>50</sub> Quantitation assay on RSV stock A2 P. Vero grown up yesterday

Made 10<sup>-1</sup> through 10<sup>-12</sup> dilution series of A2 P. Vero in Virus growth medium

-1 -2 -3 -4 -5 -6 -7 -8 -9 -10 -11 -12

4 quadruplicate repeats

→ A	.	.	.	.	.	.	.	.	.	.	.	.
→ B	.	.	.	.	.	.	.	.	.	.	.	.
→ C	.	.	.	.	.	.	.	.	.	.	.	.
→ D	.	.	.	.	.	.	.	.	.	.	.	.
E												
F												
G												
H												

VIRUS GROWTH MEDIUM ONLY

Empty ant  
growth medium  
Replace with  
200µl of diluted  
virus.

A2 P. Vero

96 well TC plate

Calculation of Titer

1 well with c.p.e. = 0.5 log of virus / 180µl  
Maybe inaccurate due to variations in cell density.

2 wells = 1 log  
3 wells = 1.25 log  
4 wells = 1.5 log

i.e. after 2 wells each  
well is worth 0.25 logs of virus

To Page No. 5

Witnessed & Understood by me,

Holly Abrams

Date

Invented by

Recorded by

David K. Clarke

Date

000027

TITLE: \_\_\_\_\_

From Page No. 4

Checked cytopathic effect of 18537 virus on vero - there is much more fusion and giant cell formation than yesterday - the 'trick' of adding fresh medium seems to have worked!

Also checked 96 well plate for TCID<sub>50</sub> results - Not yet clear, but it seems that the  $10^{-1}$  -  $10^{-3}$  dilutions are showing significant fusion activity - will leave over the weekend and do final read on Monday or Tuesday. Will check c.p.e. on 18537 later this afternoon and decide if should harvest today or tomorrow.

Prepared a fresh 1M Hepes solution (pH 7.5 with HCl) - autoclaved in 'big autoclave'.

Stored in tissue culture room

Checked c.p.e. (4pm) - progressing well - will harvest either later tonight (after dinner with Patrick) or tomorrow. TCID<sub>50</sub> looks pretty good - at least  $10^{-1}$  and  $10^{-2}$  dilutions are showing complete fusogenic activity - may indicate a titre of (assuming  $10^3$  cells/well)  $\geq 10^4$  -  $10^5$  p.f.u./ml. Will read again tomorrow and Monday.

Ordered Methyl Cellulose from VWR 1 Kg Mx08501 received. Stored in Chemicals Rm.

Gpm checked c.p.e. again - will leave until tomorrow to harvest. Will also check TCID<sub>50</sub> plate.

Harvested RSV 18537 p1 Vero - c.p.e. was not as advanced as I would have liked, but it would have been counterproductive to wait any longer. Added 1ml of Hepes pH 7.5 + 1ml of 2M MgSO<sub>4</sub> - Aliquoted and froze in 1M.

EtOH/dry ice - then put at -70°C

Checked TCID<sub>50</sub> plate - Now looks as though there is c.p.e.  $10^{-1}$  -  $10^{-3}$ . Will check again on Mon. 5 days post infection.

To Page No. 6

Witnessed &amp; Understood by me,

Date

Invented by

Date

Gally Abrams

Recorded by

David K Clarke

000028

From Page No. 5

Checked TCID<sub>50</sub> plates (Day 5 post infection) Results look very promising - approx  $10^{7.5}$  pfu ml<sup>-1</sup> (or greater!) Will make final reading tomorrow.

Tomorrow will carry out TCID<sub>50</sub> measurement on RSV 18537  $10^{-1} - 10^{-8}$

(RSV 18537 p.Vero) Will also try to boost growth of 18537 (would predict that titer will be low). To do this will infect  $1 \times 25 \text{ cm}^2$  flask with 0.5ml of undilute virus and one flask with 0.5ml of  $10^{-1}$  diluted virus. - Adsorb 2 hrs +

(600  $\mu\text{l}$  for  $75 \text{ cm}^2$  bottle) Similarly - will infect  $1 \times 25 \text{ cm}^2$  bottle with  $\approx 200 \mu\text{l}$  of undilute RSV A2 p.Vero and  $1 \times 25 \text{ cm}^2$  bottle with  $200 \mu\text{l}$  of dilute ( $10^{-1}$ ) RSV A2 p.Vero

Received  
Stored in  
chem stocks  
Ordered 100g Sigma Hepes (as a highly pure stock for careful mol. Biol. work)  
Organised a scheme for synthesising a full length cDNA for the RSV Genome

Checked RSV A2 p.Vero TCID<sub>50</sub> -

	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10
A										
B										
C										
D										

27 TOTAL SHOWING C.P.E

$$1 + \frac{26}{4} = 7.25$$

i.e. titer of stock is  $10^{7.25}$  ml<sup>-1</sup> (Pretty darn good!)

Started infection of small  $25 \text{ cm}^2$  (?) bottles

Used undilute and  $10^{-1}$  diluted inoculum for RSV A2 and RSV 18537 p.Vero

Used 0.5ml of each dilution for RSV 18537 - in an attempt to boost M.O.I. and build up titer of that stock.

Used 0.2ml of each dilution for RSV A2 p.Vero in an attempt to build

To Page No. 7

Witnessed &amp; Understood by me,

Date

Invented by

Date

Holly Abrams

Recorded by

David Clarke

0000029

TITLE \_\_\_\_\_

From Page No. 6

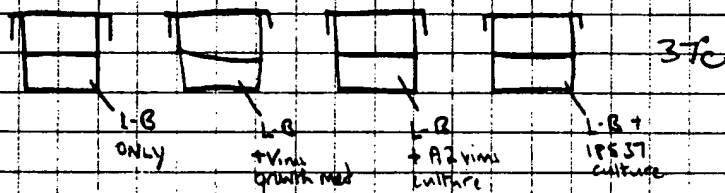
up titers even more for that stock, possibly by eliminating DI at  $10^{-1}$  or increasing MOI at midlate.

Investigator called about T/A vector - They said it should be possible to PCR up 5Kb fragments and clone them

After adsorbing virus for  $\approx 2$  hrs at  $37^{\circ}\text{C}$  added 3-4ml of virus growth medium and replaced at  $37^{\circ}\text{C}$ .

$\approx 3:30$  pm Set up TCID<sub>50</sub> on RSV 18537 p. vero  $10^{-1} - 10^{-8}$  dilutions  
Incubated on to observe c.p.e. will check again daily through the weekend

Checked c.p.e. on cell monolayers infected with RSV - 24h.p.v.  
C.p.e. is not yet distinctive - will check again later this afternoon.  
The TCID<sub>50</sub> is not showing generalized fusion (as the A2 did) at  $10^{-1}$  and  $10^{-2}$  - indicating the input titer is low.  
Will set up sterility tests on virus cultures + virus growth medium



3-45pm checked c.p.e. again on A2 and 18537 infected cells - There is advanced c.p.e. on the undilute infected cells; and quite a large amount of c.p.e. on the A2  $10^{-1}$  infected cells. There was no observable c.p.e. on the  $10^{-1}$  dil 18537 infected cells, but perhaps a little at undil 18537. Nothing is visible yet on the TCID<sub>50</sub> 18537 plate.

(NB) Should try different cell lines for RSV 18537 if yields cannot be improved on Vero.

To Page No. 7

Witnessed &amp; Understood by me,

Date

Invented by

Date

Jelly Abrams

Recorded by

David K Clarke

000030

From Page No. 7

Checked sterility tests - they are all negative (Excellent). The gritty particulate material in the culture medium must be the result of virus cytopathology.

Checked c.p.e. on Infected monolayers. The two A2 infected monolayers have been wiped out ( $10^{-7}$  + undilute). The 18537 monolayers were also showing cytopathic effect at both dilutions - will incubate them for a further 24 hrs. Will harvest the A2 virus this morning.

Harvested A2 virus - separately for each dilution - observed supernatants for presence of any possible contaminants (see above) - none were observed.

TCID<sub>50</sub> not yet showing any appreciable c.p.e.!

Left message with Judy Beeler for some more vials of RSV (Lyn Booth said, or other possible contacts)

Prepared fresh 10% (w/v) S.D.S. - autoclaved - stored in mol. biol. lab.

Final observation of c.p.e. on 18537 infected cells for the day; looks good at both dilutions - will probably harvest tomorrow.

To Page No. 9

Witnessed &amp; Understood by me,

Date

Invented by

Date

Holly Abrams

Recorded by

David K. Clarke

000031

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

10% SDS solution has turned milky; Maybe autolysing is not useful for high percentage SDS solns!!

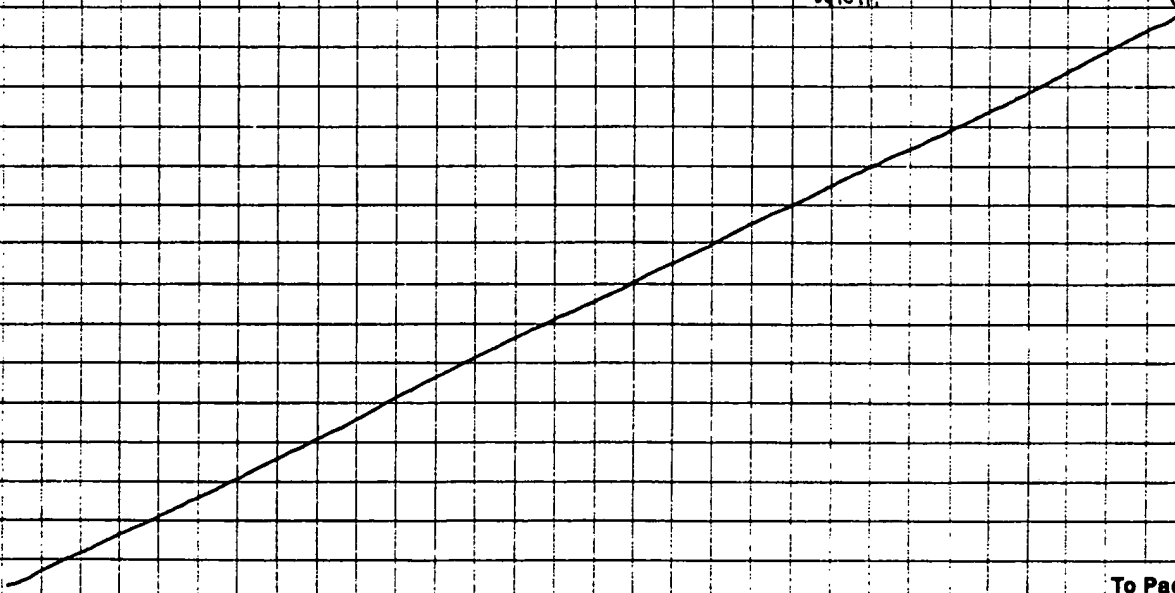
Checked TCID<sub>50</sub> (RSV 18537 p2 Vero) - c.p.e. now detectable out to 10<sup>-3</sup> dilution after 3 days; will make final reading on Monday

Chp.i Checked c.p.e. on RSV 18537 p2 Vero infected cells - undil - shows massive c.p.e. dil - shows good c.p.e. but not as extensive as the 'undil' flask

'Sterility' control is still the only one which shows any sign of contamination. This indicates that fortuitous contamination can occur; however it appears that none of my virus stocks or solutions are contaminated.

Initiated harvest of virus from RSV 18537 p2 Vero undil and 10<sup>-1</sup> dil. Followed protocol carefully - froze away in 0.5ml or 1ml aliquots as RSV 18537 p2 Vero 10<sup>-1</sup> or undil.

Carried out TCID<sub>50</sub> on RSV A2 p2 Vero 10<sup>-1</sup> + RSV 18537 p2 Vero 10<sup>-1</sup> undil. undil.



To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Holly Abrams

Date

Invented by

Date

Recorded by

David R Clarke

000032

From Page No. \_\_\_\_\_

Came in to check on TCID<sub>50</sub>s

- ① Day 5 post infection for RSV 18537 p<sub>1</sub> virus (Final reading tomorrow unlikely to change)

	-1	-2	-3	-4	-5	-6	-7	-8	-9
A									
B									
C									
D									

17 wells showing c.p.e.  $\equiv 1 + 3.75 = 10^{4.75}$ 

This value is close to the expected titer

- ② Also checked TCID<sub>50</sub>s (at 48 h p.i.) for A2 p<sub>2</sub> virus  $10^3$ /undil and 18537 p<sub>2</sub> virus  $10^{-1}$  and undiluted. It appears that for both viruses c.p.e. is occurring more rapidly and to a greater extent than at the p<sub>1</sub> passages - indicating either/or increased titers and improved growth efficiency (Final reading for these plates will be wed/thurs)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Holly Abrams

Recorded by

David K. Clarke

0000033

TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

11

From Page No. \_\_\_\_\_

Did final Read on TCID<sub>50</sub> for RSV 18537 p<sub>1</sub> Vero and did 3 day check  
on RSV A2 p<sub>2</sub> Vero 10<sup>-7</sup>/undil and RSV 18537 p<sub>2</sub> Vero 10<sup>-7</sup>/undil.

(6 day) Final reading on 18537 p<sub>1</sub> Vero = 18 wells showing c.p.e.

$$\text{titer} = 10^5 \text{ ml}^{-1}$$

RSV A2 p<sub>2</sub> Vero - already showing good c.p.e. out to 10<sup>-6</sup>/10<sup>-7</sup> at both dilutions.

RSV 18537 p<sub>2</sub> Vero - showing c.p.e. out to 10<sup>-3</sup>/10<sup>-4</sup> at both dilutions. (Perhaps a little more extensive at the same time as for 18537 p<sub>1</sub> Vero) Will check again this evening.

Ordered 2 small 25cm<sup>2</sup> bottles for tomorrow, 10x75cm<sup>2</sup> bottles for Wed and 2 x 96 well plates for Friday.

Ordered 1 TA cloning kit (20 RXN - \$24.5) Invitrogen

Ordered Taq Polymerase 100u Stratagene received - stored at -20°C

Ordered RNase/DNase free Sucrose Sigma 1kg Received - store room

Checked TCID<sub>50</sub>s once again - it appears that A2 p<sub>2</sub> Vero undil is showing extensive c.p.e. out to 10<sup>-8</sup> dilution which is a titer of 10<sup>8.5</sup>.  
The diluted A2 p<sub>2</sub> Vero is not quite as extensive.

The 18537 p<sub>2</sub> Vero undil were showing c.p.e. to 10<sup>-4</sup> - will check both again tomorrow (Day 4)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Holly Abrams*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

*Wendy & Charlie*

000034



From Page No. \_\_\_\_\_

✓  
 Checked TCID<sub>50</sub> again - There is c.p.e. at  $10^{-4}$  dilution on RSV A2 p2 Vers Undil with a total of 31 wells showing c.p.e. This is equivalent to a titer of  $10^{3.25}$ . It would appear that this pool is devoid of DIs so far, as titer is increasing with increasing M.O.I.

The RSV 18537 p2 Vers TCID<sub>50</sub> are showing some c.p.e. out to  $10^{-5}$  dilution in the 'DI' infection but not at 'undil' infection - indicating a presence of DI particles in this stock. If c.p.e. occurs at  $10^{-5}$  in all 4 wells then a titer of  $10^{5.5}$  would be indicated.

11am. Infected two 25cm<sup>2</sup> Flasks ( $\approx 100\%$  confluent) with RSV 18537 p2 Vers undil. (from TCID<sub>50</sub> this stock should be higher titer than 'undil' stock). Did one infection with 0.5ml of this stock undiluted, and for the 2<sup>nd</sup> flask infected with 50 $\mu$ l of undilute stock + 150 $\mu$ l MEM (200 $\mu$ l final vol) to give equivalence in plaque to infecting with 0.5ml of  $100 \times 100 \mu$ l ( $10^{-4}$  dil). This reduction in volume may facilitate adsorption while minimizing DI involvement.

Completed infection process at 1:45pm - added 3.5ml MEM to undil infection and 4.0ml to  $10^{-4}$  dil infection.

Checked TCID<sub>50</sub>s again. May have jumped the gun on 18537. C.p.e. may be better on undil. Will wait another day or two to find out. Meanwhile will monitor di infections set up earlier this morning.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Holly Abrams

Recorded by

David R. Clarke

0000035

TITLE \_\_\_\_\_

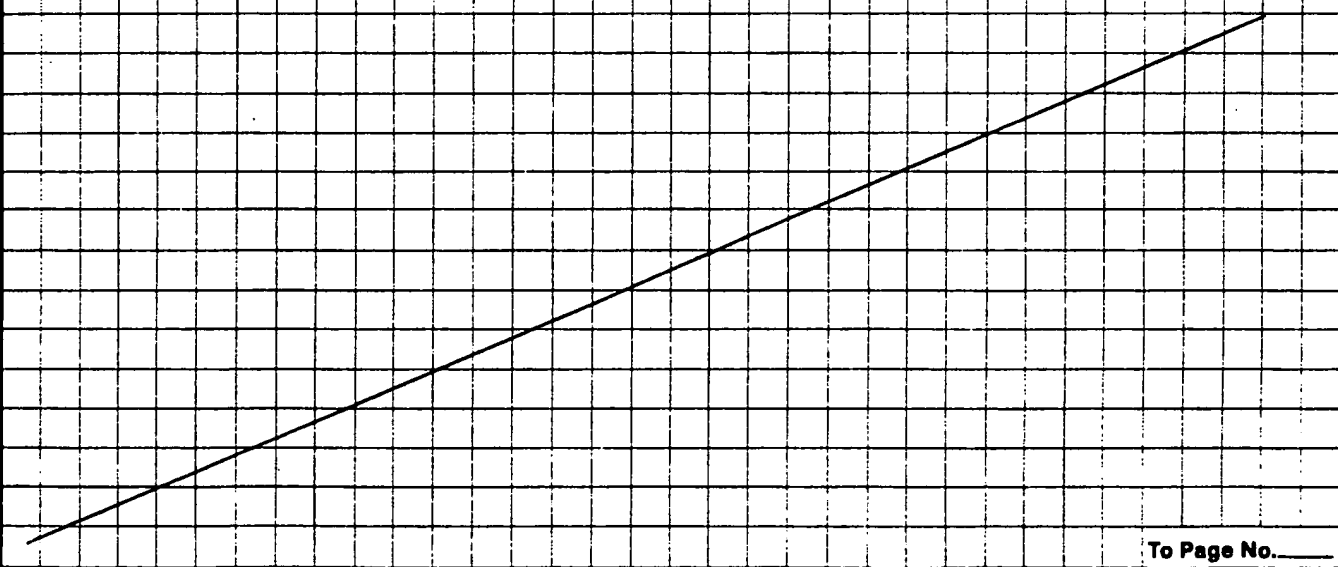
From Page No. \_\_\_\_\_

Checked Vero cells needed for production of working stock - they are not yet sufficiently confluent - will check again at  $\approx 3pm$  but most probably will wait until tomorrow to infect them.

Checked TCID<sub>50</sub>s once more :- it seems that  $10^{-1}$  dil<sup>n</sup> RSV 18537 p2 Vero is giving the highest titer (vs. undil A2 p2 Vero) - out to  $10^{-5.5}$   $10^{5.5} ml^{-1}$  (up  $\frac{1}{2}$  log). Monolayers infected with 18537 p2 Vero Dil (at undil + dil<sup>n</sup>) are already showing a little C.p.e. at 24h.p.i. especially at  $10^{-1}$  dilution. RSV A2 p2 Vero TCID<sub>50</sub> is showing good C.p.e. - especially at undil where a titer of  $10^{8.25}$  is indicated (31 wells with C.p.e.) - up  $\approx 1$  log!!

Try. Rd arrived  
from Stratagene.

Checked confluence of Vero for working stock of A2 - still not ready - will wait until tomorrow to infect to ensure max. virus production.



To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Holly Abrams

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

recorded by

David K. Clarke

0000036

From Page No. \_\_\_\_\_

Did final Read on TCID<sub>50</sub>s For RSV A2 P<sub>2</sub> Vero undil/dil - Undil gave highest titer

at

	-1	-2	-3	-4	-5	-6	-7	-8
A								
B								
C								
D								

31 wells with C.P.E. = 1+7 =  $10^{8.25}$

undil.

For RSV 18537 P<sub>2</sub> Vero

	-1	-2	-3	-4	-5
A					
B					
C					
D					

Dil - gave highest titer.

20 wells with c.p.e.

Translates into titer of 4+8 = 5

titer =  $10^{5.5}$

Checked C.P.E. on RSV 18537 P<sub>2</sub> Vero dil - infected cells - they are progressing well at both 10<sup>-1</sup> dil and undil. - will probably harvest tomorrow

checked confluence of cells to be infected with RSV A2 P<sub>2</sub> Vero (undil) - They look ready ~ 95% confluent. Will infect ~ 2-3 pm.

2-4:5pm Infected 10 x 75 cm<sup>2</sup> Vero monolayers with 0.5-0.7 ml of RSV A2 P<sub>2</sub> Vero stock (undiluted) - adsorbed for ~ 2 hrs at 37°C then fed each monolayer with ~ 10 ml of pre-warmed virus growth medium.

C.P.E. is progressing well on RSV 18537 P<sub>2</sub> Vero dil infected at 10<sup>-1</sup> and undil. Will harvest both tomorrow and do TCID<sub>50</sub>s

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Holly Abrams

HDA

Recorded by

David A. Clarke

000037

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

Checked CPE on 25cm<sup>2</sup> bottles infected with RSV 18537 p2 Vero dil/undil. Undil was showing extensive CPE. Harvested both monolayers (harvesting may not have been as efficient as usual due to confluence panic!) Aliquotted and froze away. Will do TCID<sub>50</sub> on dil/undil later today.

Checked c.p.e. on A2 RSV infected cells (had used A2 p. vero undil to prepare stock) Not extensive cpe showing yet, will check again this afternoon.

Set up a 2<sup>nd</sup> sterility control: this time used T<sub>1</sub> culture medium in 25cm<sup>2</sup> flasks, added 5ml of V. growth medium, and a drop of supernatant from RSV A2 infected cells.

Checked through CV's to find a potential RA. - there are ~ 8 promising individuals - Talked to Kathy; decided not to hire an RA for a month or two (or 3 or 4!!)

Set up TCID<sub>50</sub> on RSV 18537 10<sup>-1</sup> - 10<sup>-7</sup> Dil and Undil.  
P3 Vero Stock.

Prepared 1 liter of STE pH ~ 7. (100mM NaCl, 10mM Tris, IMMEDIA) - Autoclaved in the Tomy

35ml Ultraclear tubes for sucrose gradients; probably ~ 5ml of 60% cushion + 30-33ml of 30%.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

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Holly Abrams

Recorded by

David K. Clark

000038

From Page No. \_\_\_\_\_

Harvested large working stock of RSV A2 P<sub>2</sub> Vero - collected a total of  
≈ 100ml. Aliquotted in various sized tubes - froze in ETOH/Dry ice and stored at  
-90°C

Checked sterility test - does not appear to be anything growing.

It seems that the virus infected cells are extruding cellular material into the medium;  
it appears to be somewhat attached to adjacent cells and not free floating.

Will use working stock diluted  $\frac{1}{2}$  to make RNA (genomic).

Will need a total infecting volume of 20ml - assuming 1ml/150cm<sup>2</sup> flask.  
Will use 10ml of working stock diluted in 10ml of virus growth medium (WED).  
It may be possible to use  $\frac{1}{10}$  vol of stock, but will check this later in the week.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Holly Abrams*

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Recorded by

*David R. Clarke*

000039

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Checked sterility control - it does appear there is a contaminant present in RSV A2 stock - will try spinning it out with the microfuge + prior to preparation of RNA (genomic)

Checked TCID<sub>50</sub> (Day 3) for RSV 18537 - There is c.p.e. out to  $10^{-4}$  in  $10^{-1}$  dilution (but not at undil). Need to check passage Number of Vero's with Kim (They may becoming refractile for RSV.)

Decided to throw out all RSV A2 stocks due to yeast contamination.  
(Called Judy Becker for more RSV)

Ordered 1ml of RSV A2 from ATCC.

Will infect  $2.5 \text{ cm}^2$  Flasks with  $\left\{ \begin{array}{l} \text{RSV 18537} - 10^{-4} \\ \text{P3 Vero DI} - \text{undil} \end{array} \right\}$  will by removing supernatant after 24 hrs - 28 hrs. i.e. 2-4-5pm tomorrow  
Used 0.5ml for each infection  
Adsorbed about 2hrs - for each with 4.5ml of MEM.

prepared 60% and 30% Sucrose solutions in S.T.E. - Boiled for 15-20min to sterilise - stored at Room temp.

Went over talk with Kathy.

To Page No. \_\_\_\_\_

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Date

Holly Abrams

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David K Clark

0000040

From Page No. \_\_\_\_\_

Checked TGED<sub>50s</sub> for 18537 p3 Vero d1  $\rightarrow$  undil  
 $\rightarrow$  Dil.

C.p.e. is observable out to  $10^{-5}$  in  $\frac{2}{4}$  wells at both Dil and Undil passage

This would give a tentative titer of  $\approx 10^5 \text{ ml}^{-1}$ . Down a little from the previous titer, but acceptable as part of the overall process of building up the titer.

Checked c.p.e. on 18537 p3 vero infected cells  $10^{-7}$  and Undil - nothing significant yet - will change medium around 4-5pm this evening

Ordered 250 5ml cryovials. (Sigma)

① Long Ramp time between hybridization and extension was crucial factor.

② 10% DMSO in Taq. buffer.

③ 1bu  $100 \mu\text{l}^{-1}$  Taq. Pol. pH?  
Mg?

④ fairly long denaturation, annealing and RXN times.

Recommendations for PCR if problems arise: à la Laurent Roux.

T<sub>7</sub> prefers to start an RNA

chain with a G but A works too.

⑤ Laurent transfected in plasmids containing DI construct (linearized with BamI) also, NP, P/C and L genes into vaccinia infected cells. Helper ~~photo~~ not work

To Page No. 18

Witnessed &amp; Understood by me,

Holly Abrams

Date

Invented by

Date

Recorded by

David K. Clarke

000041

TITLE \_\_\_\_\_

From Page No. 18

3pm. Replaced virus growth medium from "18537 p. 5 Var. Dil." infected cells ( $10^4$  and undil.)

and replaced with 4ml of fresh virus growth medium (in an attempt to further boost titer of virus stock)

Checked TCID<sub>50</sub> again - no change - looks like  $10^5$  will be final titer - but will check again tomorrow and then

Ordered a fridge-freezer (probably from Sears)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Holly Abrams*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by

*David R. Clarke*

0000042



From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub> for RSV 18537 P<sub>3</sub> Vero Dil/undil stocks (Day 5) Can  
probably conclude titers today

	-1	-2	-3	-4	-5	-6	-7	-8
A								
B								
C								
D								
A								
B								
C								
D								

10<sup>5</sup> ml<sup>-1</sup>

Dil.

This titer is a little down from the P<sub>3</sub> Vero  
titer; however will continue to passage this  
virus in an attempt to bring up titer from  
10<sup>5</sup> → 10<sup>6</sup>

Undil

10<sup>5</sup> ml<sup>-1</sup>

Also checked c.p.e. on '18537 P<sub>3</sub> Vero Dil' - infected  
cells; 10<sup>5</sup> and undil. c.p.e. is pronounced on both -  
perhaps a little ahead on the undil infection - might  
harvest it tonight and 'dil' tomorrow morning.

~10am Infected 1 x 75 cm<sup>2</sup> of Vero with 10.5 ml of RSV A2 P<sub>3</sub> Vero (undiluted) - This virus was  
saved from the initial growth of RSV A2 prior to addition of MgSO<sub>4</sub> / Hepes. I wanted to  
check if the yeast contamination was present in that stock. If so, it would indicate  
that the contamination came in with the stock.

Virus arrived from Judy Beeler - stored at -70°C

2pm checked c.p.e. again on undil 18537  
and harvest the dil tomorrow morning

- Decided to harvest this evening

Stored at -90°C  
as 18537 P<sub>4</sub> Vero undil.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Holly Abrams

Date

Invented by

Recorded by

David R. Clark

Date

000043

TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

21

From Page No. \_\_\_\_\_

Scientific presentations all Day

Sam Harvested RSV 18537 P. van Dil - aliquotted and froze down at  $-90^{\circ}\text{C}$ .  
C. pe. looked quite eduried.

Scientific Meeting

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Holly Abrams

Date 4/10/81

Invented by

Date

Recorded by

David K Clarke

0000044

From Page No. \_\_\_\_\_

Checked c.p.e. on 'A2 p. Vero' infected Vero - looked advanced, so decided to harvest. Also noticed that the contaminating yeast is still there, indicating that it was probably not introduced by me and probably came in with the stock we received from the FDA, ~~etc~~ and in the absence of Fungizone was able to replicate easily.

### Notes on Attenuation of RSV :-

- ① Manipulation of N gene - a) level of expression - RNA or protein.  
     b) functional domains - which interacts with RNA.  
     To give reduced replication, yet good transcription  
     Must be sufficient replication to allow rescue.  
     (May need to use stably transformed cell line to supply N protein in trans, to allow replication of seed vaccine)
- ② Alter cytoplasmic domains of G and F to give reduced rate of migration through ER and Golgi
- ③ Eliminate expression of NS1, NS2, M2, SH to observe effects on virus growth and virulence.
- ④ Mutate M, F, and G genes to give slightly reduced expression of these proteins, to slow down maturation
- ⑤ Reduce number of Lys/Arg residues at cleavage site in F protein to reduce efficiency of cleavage
- ⑥ Manipulate transcription and and/or replication regulatory sequences

To Page No. 22

Witnessed &amp; Understood by me,

Holly Abrams

Date

Invented by

Date

Recorded by

David K. Clarke

000045

TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

23

From Page No. 22

⑦ Alter part of (M) protein involved in switching off of host transcription - without affecting assembly

Made preparations for TCID<sub>50</sub>s before 2pm meeting with Peter Palese. Will make dilutions + infections later today

Set up TCID<sub>50</sub>s on 'A2 p2 Vero until'  
18537 p<sub>2</sub> Vero dil/untill

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date ✓

Invented by

Date

Holly Abrams

HOA

Recorded by

David K Clarke

0000046

From Page No. \_\_\_\_\_

Day 3. Checked TCID<sub>50</sub>s - There is c.p.c. out to  $10^{-4}$  in Undil 18537 P<sub>1</sub> Vero.  
out to  $10^{-6}$  in RSV A2 P<sub>2</sub> Vero

Will infect 25cm<sup>2</sup> bottles with "Undil 18537 P<sub>1</sub> Vero" and dil.

" " " " with New 18537 (from Judy Beeler) dil/Undil } 0.5 ml each infection

" " " " with New A2 undil and dil.

Ham did infection - adsorbed 2 hrs at 37°C.

Prepared some fresh virus growth medium ABG +, Glc +, NEAB +, FCS - heat inact +.

RSV A2 Vero p<sub>2</sub> may not be giving as high a titer this time around for a number of reasons

- ① There was no Hepes/MgSO<sub>4</sub> in the supernatant - used to stabilize the virus
- ② Vero cells may be are becoming refractile to RSV (at higher passage No.)
- ③ Cell monolayers were a little sparse

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Holly Abrams

Date

HOK

Invented by

Recorded by

David K Clarke

Date

0000047

TITLE: \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

25

From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub>s for 8537 p<sub>2</sub> Vero dil/untill and A2 p<sub>2</sub> Vero

It seems that there are 3 wells showing c.p.e. at  $10^{-5}$  dil<sup>n</sup> on 8537 p<sub>2</sub> Vero untill.  
which would translate into a titer of  $10^{5.25}$ . In A2 p<sub>2</sub> Vero - there is c.p.e. out to  
 $10^{-7}$  dil<sup>n</sup>  $\approx 10^{7.5}$  Will make final read-out tomorrow morning

Checked cytopathic effect on all three sets of infections - there are already some  
indications of c.p.e. on some of the monolayers - especially the one infected with  
A2 pp3D untill

Ordered 2,500 units of Ribavirin from Promega \$175.

Did final check on c.p.e. for today - looks as though there is already  
recognizable giant cell formation in most of the infections - will harvest either  
tomorrow morning or this morning

Witnessed &amp; Understood by me,

Name

Holly Abrams

Invented by

Date

Recorded by

David K Clarke

HDA

To Page No. \_\_\_\_\_

000048

From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub> for final read

-1 -2 -3 -4 -5 -6 -7 -8



18 wells  
 $= 10^5 \text{ pfu ml}^{-1}$

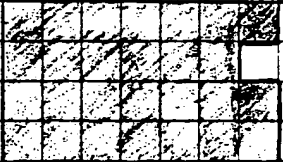
18537 p<sub>2</sub> Ven

Dil

19 wells  
 $= 10^{5.25} \text{ pfu ml}^{-1}$

Undil

-1 -2 -3 -4 -5 -6 -7 -8



27 wells  
 $= 10^{7.25} \text{ pfu ml}^{-1}$

RSV A2 p<sub>2</sub> Ven

Undil

Checked cytopathic effect on infected 25 cm<sup>2</sup> Flasks

① \* RSV A2 (New viral from J. Beeler) - Dil - Good c.p.e. at 48h p.i.

Undil - Buble interference but cells look sick  
will leave a 3<sup>rd</sup> day.

\* The 'gaungies' observed in the 1<sup>st</sup> vial  
 of this stock are again present,  
 indicating J. Beeler's stocks are  
 contaminated with either yeast/fungus  
 or penicillin resistant bacteria.

② RSV 18537 pp2 new stock of 'R. virus' from J. Beeler.  
 Not much c.p.e. yet at 48h p.i. - will change medium for  
 shock treatment to boost virus production.

③ RSV 18537 p<sub>2</sub> Ven Dil - c.p.e. at both dilutions - will  
 leave for a 3<sup>rd</sup> day.

To Page No. 27

Witnessed &amp; Understood by me,

Date

Invented by

Date

Hally Abrams

Recorded by

David K. Clatter

0000049

TITLE \_\_\_\_\_

From Page No. 26

6pm Checked cpe again. 2A infected cells are showing gross cpe - greatest at 'dilute' - equivalent to 50µl of infective stock. Will use 150-200µl of stock (J. Geelers) + 300µl MEM containing Fungazone to infect a 75cm<sup>2</sup> bottle.

Need to add 1ml Fungazone to 500ml MEM or 0.1ml/50ml or 50µl/25ml

Will infect 1 x 75cm<sup>2</sup> bottle with 0.5ml of ATCC Riv A2.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Holly Abrams*

Recorded by

*David K Clarke*

000050



From Page No. \_\_\_\_\_

Checked c.p.e. on infected cell monolayers

A2 - P. Verm 2<sup>nd</sup> batch Dil. } Cells blown away. complete c.p.e.  
Undil.

1<sup>st</sup> batch 18537 P<sub>5</sub> Verm Dil. } Cells blown away - complete c.p.e. in Undil. - little less c.p.e.  
Undil. } in dil.

Harvested these four flasks separately and freeze down cells at -90°C

Will infect 1 x 75 cm<sup>2</sup> Flask with 200 µl of A2 (+300 µl MEM) - from ~~from~~ Judy Reeler  
pp3D Hep 4 in presence of Fungizone (50 µl/25 ml)

Will infect 1 x 75 cm<sup>2</sup> flask with 0.5 ml of ATCC A2 virus

⊙ T/A cloning kit from Invitrogen received.

Absorbed virus for ~2 hrs at 37°C

Ordered cells for next week.

5<sup>th</sup> day harvest! Carried out TCID<sub>50</sub> on A2 P. Verm 2<sup>nd</sup> Batch (also contains "ganglions") as  
a matter of interest. Dil + Undil.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Holly Abrams

recorded by

Darwin R Clarke

000051

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Checked  $TCID_{50}$  of A2 P. Vero 2<sup>nd</sup> batch (contaminated). Not yet any significant c.p.e.

4<sup>th</sup> day checked 18537 P. Vero (New) - Not showing dramatic c.p.e. Will harvest until today and run a  $TCID_{50}$  on Mon

Checked A2 infected cells - J. Becker 3<sup>rd</sup> batch + Fungazone.  
- ATCC virus

Both monolayers are showing significant c.p.e. at 2<sup>th</sup> p.i. Will harvest either on Sat or Sun

Dr. Kevin Moore (ELI) Gave a seminar at lunch time

Infected 2 x 25cm<sup>2</sup> flasks with 18537 P. Vero (300µl) - one flask with virus from Dil. P. Vero - one flask with virus from undil. P. Vero - Adsorbed  $\approx$  2 hrs (used undil. medium in both cases)

Did  $TCID_{50}$  on 18537 P. Vero Dil. undil.

Harvested 18537 P. Vero (New batch) dil and though cytopathology was not very significant. until freeze and stored in Upright freezer - 2<sup>nd</sup> shelf from the bottom

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Hally Abrams

Date

Invented by

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Recorded by

David K Clarke.

0000052

From Page No. \_\_\_\_\_

Checked cpe on RSV A2 infected cells. p, Vero

ATCC } cpe v extensive on both.  
J. Becker }

Could still see contaminating bug in J. Becker's prep.  
assume it is Pen<sup>R</sup>/Strept<sup>R</sup> + Fung<sup>R</sup> Will filter sterile virus harvest and back titers back  
up if there is a loss.

Harvested both flasks of virus - Freeze at -90°C (bottom tray)

Checked cpe on TCID<sub>50</sub> <sup>J. Becker's</sup> RSV A2 p, Vero Dil/Unadil; cpe visible out to 10<sup>-5</sup>.

No appreciable cpe yet on RSV p, Vero.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Holly Abrams

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David K. Clarke

Date

000053

TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

31

From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub> of ~~(18537)~~ A2 p<sub>1</sub> Vero 2<sup>nd</sup> batch - Undil gave highest  
titer  $\approx 10^{5.50}$  - will make final reading tomorrow.

Checked C.P.E. on 18537 p<sub>5</sub> Vero Dil } infected 25cm<sup>2</sup> bottles - C.P.E. is  
Undil }

not as extensive as I would have  
anticipated - it appears the cells are becoming refractile to virus growth.

Will wait for newer cells to start cloning work (next morn.) meantime will  
harvest 18537 p<sub>6</sub> Vero later today for comparison with similar 3 day infection with  
newer cells.

Infected 2 x 75cm<sup>2</sup> Flasks with { A2 p<sub>1</sub> Vero Undil ATCC  
A2 p<sub>1</sub> Vero Undil J. Becker }

Adsorbed  $\approx 2$  hrs. at 37°C.

\* Slightly lighter cell monolayer.

Set up TCID<sub>50</sub> on A2 p<sub>1</sub> Vero ATCC } make up til after 10<sup>-5</sup> dil - shouldn't  
J. Becker } be a problem though.

(3 days p<sub>1</sub>) Harvested virus from 18537 p<sub>6</sub> Vero Dil/undil - freeze down for  
comparison with p<sub>6</sub> Vero from younger cells.

Checked TCID<sub>50</sub>s again - still looks as though there is a reduction in the  
number of viruses produced - possibly due

May go back to 18537 p<sub>2</sub> Vero when the newer cells are ready in order to  
try and boost titers even more (from the max of 10<sup>5.5</sup> observed for p<sub>2</sub> Vero 18537)

⊗ Harvesting a little early - rather than waiting for total destruction of cells may  
also be important

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Hally Abrams*

Recorded by

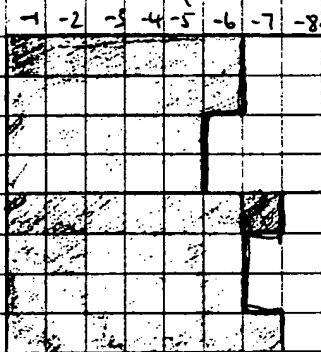
*David K. Clarke*

0000054

## Analysis of cDNA (ss.) by Atk. gel elect. is in sect<sup>n</sup> 6.2 Mania to.

Checked TCID<sub>50</sub>s. A2 p. varn 2<sup>nd</sup> batch - titer appears to be 6.75 on 10

undil (lower undil)



106

D1.

RSV A2 P. Vero 2<sup>nd</sup> batch (J Baker)

10

Unit 1

Checked TCID<sub>50</sub> of 18537 p5 Vero - There is c.p.e. out to  $10^{-4}$  in some cases - however there appears to be some drop in titer - again possibly reflecting refractility of Vero cells. (Kim says they are  $\approx 14$  passages out from frozen - should change at 10-12 passages.)

I am looking forward to comparing New/old cell titers with 18537p5 Vacc

## Important Notes

- ① Use young cells  $\Rightarrow$  0 passages from frozen.
- ② Adsorb virus in small volume for 2-3 hrs with high M.O.I. as possible.
- ③ Time of harvest may be important; e.g. too late may result in drop in titer due to virus  $\frac{1}{2}$  life.

May go back to 18537 p<sub>2</sub> Vero (which had 0.5<sup>+</sup> titer) in order to try and boost titers (next week).

To Page No. 30

**Witnessed & Understood by me,**

**Date****Invented by****Date**

Holly Abrams

**Recorded by**

Recorded by Daniel K. Clarke

000055

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

33

TITLE \_\_\_\_\_

From Page No. 32

5:20 pm checked c.p.e. on RSV A2 p<sub>2</sub> Vena T<sub>2</sub> later, again. Cpe appears to be  
ATEE.

- progressing reasonably well - will check again tomorrow morning.

Witnessed & Understood by me,

Date

Invented by

Date

To Page No. \_\_\_\_\_

*Steve Kibbe*

scored by

*David K Choche*

000056

From Page No. \_\_\_\_\_

Checked A2 infected cells - cpe is well advanced in ATCC infected cells (p2Vero) and in J. Beeler infected cells - may harvest this evening or tomorrow morning. Will filter sterilise J. Beeler stocks and include tetracycline in the growth medium.

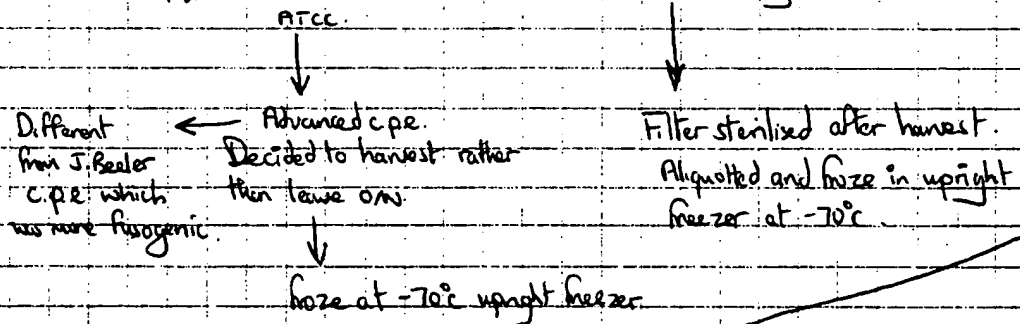
Did final reading on RSV A2 p. Vero 2<sup>nd</sup> Batch J. Beeler - results indicate a titer of  $10^7$  units.  
 $10^6$  dil.

Checked TCID<sub>50</sub> of 1B537 p. Vero - tomorrow will make a final reading. It appears there is cpe out to  $10^{4.4}$  in Dil (21 wells would be a titer of  $10^{4.25}$ ) - down on previous titers. It looks as though the cells are refractile (more so) than during earlier passage N°.

2 days Pk. There is not much information available on TCID<sub>50</sub> for A2 p. Vero J. Beeler 2<sup>nd</sup> Batch + ATCC Virus

Carried out TCID<sub>50</sub> on 1B537 p. Vero 2<sup>nd</sup> Batch - don't expect much of a titer

Harvested A2 p2Vero J. Beeler - Extensive cpe. - but heavily contaminated.



To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Steve W. Kuhl*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

*David K. Clarke*

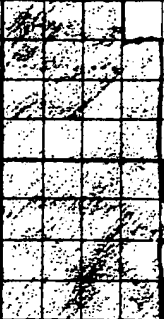
0000057

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Do final check on TCID<sub>50</sub> on 18537p<sub>5</sub> Vero 1<sup>st</sup> Batch.

-1 -2 -3 -4 -5 -6 -7 -8 -9



4.25  
10

undil

Rev 18537p<sub>5</sub> Vero

17 = 4.75  
10

D1

Overall impression that  
cell refractivity is acting  
on virus growth.

Checked CPE on TCID<sub>50</sub> for RSV A2 P<sub>1</sub> Vero - ATCC  
- J. Becker

CPE in both out to 10<sup>-4</sup>  
v. different in nature - fusogenic  
in J. Beckers - Great cell  
formation with ATCC strain.

checked TCID<sub>50</sub> for 18537p<sub>5</sub> Vero 2<sup>nd</sup> batch. - No sign of any CPE yet.

Ordered cells for next week

For Mon 2 x 96 well plates; One to compare 18537p<sub>5</sub> Vero titers  
One to check A2 P<sub>2</sub> Vero stocks - ATCC / J. Becker

1 x 25cm<sup>2</sup> Flask To do Vero cell cloning

3 x 75cm<sup>2</sup> Flasks - One to amplify 18537p<sub>2</sub> Vero (highest titer so far for  
in newer cells to try and boost B subtype)  
titer.

Two for A2 growth (Either D1/undil of one stock or  
undil of both stocks)

For Thur 4 x 75cm<sup>2</sup> Flasks to prepare working stock of either ATCC or J. Becker A2

2 x 96 well plates - one to titer A2

One to check titer on 18537p<sub>2</sub> → P<sub>3</sub> Vero titer

To Page No. 36

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Serge Kille*

Recorded by

*David K. Clarke*

0000058



From Page No. 35

Set up TCID<sub>50</sub> on RSWA2 P<sub>2</sub> Vero - get slight mix up in Dilutions but will be able to differentiate ARCC and J.B. order now based on c.p.e. unless removal of bacterial contaminant affects c.p.e.

Witnessed &amp; Understood by me,

*Sege Kiki*

Date

Invented by

Date

Recorded by

*David K. Clatter*

To Page No. \_\_\_\_\_

000059

Jodie and Paulette Leuve

Project No. \_\_\_\_\_

TITLE: \_\_\_\_\_

Today

Book No. \_\_\_\_\_

37

From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub> Day 4 for A2 p<sub>2</sub> Vero ATCC - c.p.e. to 10<sup>-4</sup>  
J. Beeler - c.p.e. to 10<sup>-5/-6</sup>

TCID<sub>50</sub> for 18537 p<sub>2</sub> Vero 2<sup>nd</sup> batch - No c.p.e. yet visible - virus  
grew in v. sickly way, so perhaps  
there were v. few virus produced.

Infected 75cm<sup>2</sup> Flasks - One with 0.5ml of ATCC A2 p<sub>2</sub> Vero  
one with 0.5ml of J. Beeler A2 p<sub>2</sub> Vero - Filter sterilized  
Adsorbed  $\approx$  2-3hrs Added 10ml of MEM containing Tetracycline ( $\frac{1}{250}$   
of stock)

No c.p.e. yet visible on p<sub>2</sub> Vero A2 TCID<sub>50</sub>.

2-Spms infected 2 x 25cm<sup>2</sup> Flasks with 18537 p<sub>2</sub> Vero undil. Made a 10<sup>-1</sup> dil<sup>n</sup>  
and infected one flask with 0.5ml 10<sup>-1</sup> dil<sup>n</sup> and one flask with 0.5ml undil.  
Used new Vero cells to try and get a comparison with older Vero (with respect to  
filter).

Checked CO<sub>2</sub> Incubator temp./CO<sub>2</sub> conc. - changed H<sub>2</sub>O.

↓  
Adjust measure on mon after CO<sub>2</sub>/Temp  
equilibrates.

Witnessed & Understood by me,

Date

Invented by

Date

*Serge Kibbi*

Recorded by

*Daniel K. Clarke*

To Page No. \_\_\_\_\_

0000060

From Page No. \_\_\_\_\_

2pm

Checked Infected cells + TCID<sub>50</sub>

A2 (ATCC) infected cells (P<sub>2</sub> Vero) until were wiped out. 24 hpi. Harvested these cells/virus and stored at -80°C in Upright freezer.

A2 (J Beeler - filter sterilized) (P<sub>2</sub> Vero) until - no c.p.e. at 24 hpi - Either all the virus stuck to the filter, or all the c.p.e. seen previously was bacterial induced. There was no sign of the bacterial contaminant.

I will try spinning some previous P<sub>2</sub> Vero A2 J Beeler in the microfuge 2 x 10 mins - and infect in the presence of tetracycline.

Checked TCID<sub>50</sub> for P<sub>2</sub> Vero ATCC/J Beeler A2

-1	-2	-3	-4	-5	-6	-7	-8		
								ATCC P <sub>2</sub> Vero	$\approx 4.5$ 10
								J Beeler P <sub>2</sub> Vero 3 <sup>rd</sup> Batch	Contains contaminant: 10 <sup>6.25</sup> ... invalid

Checked TCID<sub>50</sub> P<sub>2</sub> Vero ATCC/J Beeler until

ATCC virus is showing improved titer - will make a final reading on mon or Tues

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge Kibbe

Recorded by

David K. Clarke

000061

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Checked 18537 p5 Vero infected cells (New cells) The c.p.e. is v. extensive at  
Undil - not so extensive at dil.  
Harvested both - freeze at  $-80^{\circ}\text{C}$

Carried out TCID<sub>50</sub> on 18537 p5 Vero Dil/Undil 'New cells' to get a  
comparison with the titer from older cells

Also did of TCID<sub>50</sub> for A2 ATCC P3 Vero

Checked TCID<sub>50</sub> for A2 P2 Vero ATCC - c.p.e. out to  $10^{-5} \text{ dl}^{-1} \equiv 10^{5.5} \text{ titer ml}^{-1}$   
" " for 18537 2nd batch (70000) c.p.e. out to  $10^{-3} \text{ dl}^{-1} \equiv 10^3 \text{ titer}$   
Will make some final readings tomorrow

Infected  $1 \times 75 \text{ cm}^2$  Flask with A2 P2 Vero (1st batch) Undil after spinning  $\approx 1.2 \text{ ml}$   
for 10 mins  $\rightarrow$  1 ml spun for a further 5 mins  $\rightarrow$  used 0.6 ml to infect a  
monolayer (cells were not v. confluent  $\approx 50\%$ )

Infected  $1 \times 75 \text{ cm}^2$  Flask with A2 ATCC P3 Vero Undil (used 0.6 ml)

Infected  $1 \times 75 \text{ cm}^2$  Flask with 18537 p2 Vero (went back to older stocks) - which  
had a high titer ( $10^{5.5}$ )  $\approx 0.5 \text{ ml inoculum}$

Ordered 500u of Life Sciences Avian Reverse Transcriptase XL

Kim set up Vero cell clearing for me  $100 \text{ cells}/10 \text{ ml MEM} \equiv 1 \text{ cell}/100 \mu\text{l}$

Completed measurements of  $\text{CO}_2$  etc on incubators in Resp Virus lab.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Sege Kibbe*

Date

Invented by

Date

Recorded by

*David K. Clarke*

0000062

From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub>s on RSV A2 ATCC / J. Boaler p<sub>2</sub> Vero  
 RSV 18537 p<sub>1</sub> Vero 2<sup>nd</sup> batch

-1 -2 -3 -4 -5 -6 -7 -8

10<sup>3.25</sup>

Dil

RSV 18537 p<sub>1</sub> Vero 2<sup>nd</sup> batch10<sup>2.75</sup>

Undil

Perhaps there are a lot of DIs  
 Most probably will not proceed with this stock of  
 18537 as I already have quite a good growing  
 stock

-1 -2 -3 -4 -5 -6 -7 -8

10<sup>5.5</sup>ATCC undil. p<sub>2</sub> Vero

J. Boaler Undil (after sterilised)

Checked Infected cells Only ATCC infected (now p<sub>2</sub> Vero) - Undil - cells are showing c.p.e.,  
 they should be ready to harvest tomorrow

Took a look at Vero clones (single cell stage) - There are clearly some wells containing  
 only one cell - in some instances there were more than one cell - and in one case  
 one cell was undergoing cell division

Checked TCID<sub>50</sub>s for ATCC p<sub>2</sub> Vero - 24h p.i. - too early for meaningful assessment

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sey Kibbe

Recorded by

David K. Clarke

000063

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

Checked infected cells - ATCC plasmids are showing extensive C.p.e. - will

harvest them this afternoon - no C.p.e. yet on J. Reeler infected cells (after super was spun for 15 min in microfuge). All virus appears to be cell associated.

C.p.e. has begun on 18537 p Vero-uninfected cells - but it will probably be at least another day before they are ready for harvest.

Reverse Transcriptase arrived - stored at  $-80^{\circ}\text{C}$  in Vaport freezer

Harvested ATCC Virus p<sub>1</sub> Vero - aliquotted and stored at  $-80^{\circ}\text{C}$ . There may be a slight reduction in C.p.e. relative to the ATCC p<sub>2</sub> Vero - possibly due to DE particles - however this

On looking over previous papers on RSV sequencing (genomic RNA) a number of things can be concluded.

① Can expect  $\approx 20 \mu\text{g}$  of genomic RNA from  $\approx 20$  roller bottles  $\approx 280 \text{ TC}$  175 cm<sup>2</sup> bottles

Need RNase  
A.  
and microcentrifuge

② Can expect a high level of cellular RNA/DNA contamination - may want to add RNase A, DNase to super prior to spinning out virus either while still in MEM or prior to loading on sucrose gradient

Need formaldehyde  
denatured formaldehyde  
Filter  
Close containing RSV RNA

③ Should probably analyze a portion of each genomic prep on either a 3% Agarose gel (see D. Star) and silver stain; or on a 1% Mops/Formaldehyde gel and blot + probe

Order  
clonings

④ Analyze each synthesis on hydroxide gel with markers (need  $^{32}\text{P}$  or  $^{35}\text{S}$ )  
Use equiv. of 1-2  $\mu\text{g}$  so have RNA remaining for cloning

⑤ Set up PCR conditions carefully - taking all available knowledge into account.

To Page No. 42

Witnessed & Understood by me,

Date

Invented by

Date

*Sege Kibbe*

Recorded by

*David A. Clarke*

000064

From Page No. 41

## Calculation of virus inoculum size for infection of Roller bottles.

Surface area calculations indicate that 11.33x inoculum will be required for a roller bottle vs a 75cm<sup>2</sup> bottle.

Total inoculation vol. will be  $\approx$  5ml - can probably dilute working stock  $\frac{1}{10}$  for infection i.e. 0.5 + 4.5ml  $\rightarrow$  1 roller bottle. However a smaller inoculation vol. may be possible due to the rolling nature of adsorption.

Ordered: Potensarek }  
 DNase I } Buehlinger  
 Micrococcal Nuclease }

Did final check on 18S37 p. Virus infected cells - c.p.e. has progressed but will not be ready for harvest until tomorrow afternoon.

Need to check that we have the facility to handle 20 roller bottles worth of supernatant.

Witnessed &amp; Understood by me,

Date

Invented by

Date

To Page No. \_\_\_\_\_

Sege Kibiki

Recorded by

David R. Clarke

000065

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Checked c.p.e. on 18537 p<sub>2</sub> Vero infected 'New cells' - 3 days p.i.  
 The c.p.e. was very extensive so decided to harvest - froze down as 18537 p<sub>3</sub> Vero  
 will do a TCID<sub>50</sub> on this stock this afternoon along with  
 ATCC p<sub>4</sub> Vero stock (harvested yesterday)

Checked TCID<sub>50</sub> - will make final readings in 1-2 days time

Checked Vero clones - marked off those containing one clone only. Looks as though  
 there will be about 40 Vero clones total

Infected 4 x 75cm<sup>2</sup> flasks with ATCC p<sub>4</sub> Vero 0.5ml x 2 (10<sup>-1</sup> dil)  
 0.5ml x 2 (undil)

Did TCID<sub>50</sub> for ATCC p<sub>4</sub> Vero and 18537 p<sub>3</sub> Vero 'New cells'

Interviewed Peter Brown

Ordered cells for next week

MON - 4 x 75cm<sup>2</sup> bottles (for more ATCC virus?)  
 2 x 25cm<sup>2</sup> bottles (for more 18537 virus?)

Thur 2 x 96 well plates

Fri 20 Roller bottles

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sege W Khl*

Recorded by

*David K Clarke*

000066



From Page No. \_\_\_\_\_

Checked ATCC TCID<sub>50</sub> p<sub>5</sub> Vero - In some cases c.p.e. was visible out to 10<sup>-6</sup> indicating a titer close to 10<sup>6</sup>.

Checked RS37 TCID<sub>50</sub> p<sub>5</sub> Vero (for comparison with older cell titer) - Appears to be c.p.e. out to 10<sup>-5</sup> in some cases - indicating that the newer cells make a real difference in growth capabilities for RSV.

Checked ATCC infected cells (p<sub>5</sub> Vero d/Urdil) - No significant c.p.e. yet; however it is only ~ 20h.p.i. - will check again late this afternoon. May have to harvest tomorrow.

Checked Vero cell clones - they are growing quite well; will refed on Mon with 100-150  $\mu$ l of fresh growth medium.

Fridge - Freezer arrived from Sears - installed in My/ying's lab.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sege Kable*

Recorded by

*David K. Clarke*

000067

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

Checked c.p.e. on ATCC infected P<sub>3</sub> Vero. Not very extensive c.p.e. at 48 h.p.i. - something is wrong. Either the adsorption period was insufficient or there has been significant build up of DT particles (latter is most likely since this virus has been passaged until 5 series). Will go back to P<sub>2</sub> or P<sub>3</sub> Vero to prepare working stock.

3 hr. adsorption } Will infect each 75 cm<sup>2</sup> with 0.5 ml of ATCC P<sub>2</sub> Vero → working stock.  
Will infect each 25 cm<sup>2</sup> with 0.2 ml of ATCC P<sub>3</sub> Vero 10<sup>-1</sup>/undil.

If c.p.e. look good at 10<sup>-1</sup> dil<sup>n</sup> of P<sub>3</sub> Vero - will use that dil<sup>n</sup> to infect roller bottles.

Checked TCID<sub>50</sub> for ATCC P<sub>3</sub> Vero and 18537 P<sub>3</sub> Vero (New cell) - will make a

final reading on Monday, but it looks as though the <sup>new</sup> titers will be 10<sup>6.25</sup> and 10<sup>5.25</sup> respectively. This represents a  $\frac{1}{2}$  log improvement for 18537 - simply by using newer cells; it will be interesting to see what the 18537 P<sub>3</sub> Vero (New cell) titers will be.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge Kille

Recorded by

David K. Clarke

0000068

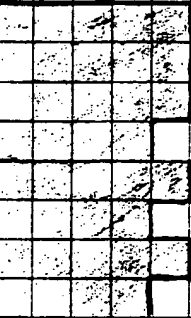
From Page No. \_\_\_\_\_

Made final TCID<sub>50</sub> readings

1 -2 -3 -4 -5 -6 -7 -8

10<sup>6.25</sup>ATCC virus  
p3 Virus Undil.This is the highest titer for ATCC virus  
so far - probably helped by the 'New cells'

1 -2 -3 -4 -5 -6 -7 -8

10<sup>5.25</sup>10<sup>5</sup>18537 Virus  
P<sub>3</sub> Virus (New cells)The dilute virus gave slightly  
higher titers this time and the  
previous time (Oct 7<sup>th</sup>). The  
Newer cells gave  $\approx \frac{1}{2}$  log higher  
titer than the older cells used on 7<sup>th</sup> OctChecked TCID<sub>50</sub> for ATCC P<sub>3</sub> Virus Undil. - appears that titer is not as high as P<sub>3</sub> Virus ATCC  
indicating the presence of D<sub>2</sub> particles. Probably should have used diluted P<sub>3</sub> Virus for infectionWill make a final reading on 18537 P<sub>3</sub> Virus "New cells" on Wed.10-30am Infected 4 x 75cm<sup>2</sup> Flasks with  $\frac{1}{2}$  diluted (0.5ml) P<sub>3</sub> Virus ATCC.Infected 2 x 25cm Flasks with ATCC P<sub>3</sub> Virus 10<sup>5</sup> dil and Undil. (200µl for each infect<sup>n</sup>)  
adsorbed 3hrs (added 1ml Hepes buffer to MEM & 20ml to stabilize pH.)Checked Vero cell clones - many cells appear to have died off - perhaps I had them  
out of the incubator too long (pH or temp affected). Will feed with some fresh growth  
medium this afternoon

To Page No. 47

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sey K. Kuli

Recorded by

David K. Clarke

0000069

TITLE: \_\_\_\_\_

From Page No. 46

Aspirated off some of the medium from the cloned Vero cells - replaced it with 150µl of fresh growth medium.

Staining RNA after transfer to nylon membrane/nitrocellulose.

- ① Soak dried filter in 5% Acetic acid for 15 mins at room temp
- ② Transfer the filter to a soln. of 0.5M NaOAc (pH 5.2) and 0.04% methylene blue for 5-10 mins at r temp
- ③ Rinse the filter in H<sub>2</sub>O for 5-10 mins. RNAs used as MW standards should appear as sharp bands.

AK Horn and Schmidt (1988). Rapid, reversible staining of N. blots prior to hybridisation. *Biotechniques* 6: 196

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sgt Kille*

Recorded by

*David K Clarke*

000070

From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub>s - ATCC p<sub>1</sub>Ver<sub>0</sub> - sample only to  $10^{-4}$  or so indicating heavy buildup of DE particles, reducing titer. Also 18577 p<sub>3</sub>Ver<sub>0</sub> (New cell) is indicating titer of  $\approx 10^5$  - a little lower than the original  $10^{5.5}$  p<sub>3</sub>Ver<sub>0</sub>. Will make final readings of these TCID<sub>50</sub>s tomorrow.

Checked p<sub>2</sub>Ver<sub>0</sub> and p<sub>3</sub>Ver<sub>0</sub> (ATCC) infected cells - just beginning to show c.p.e. - will check again late this afternoon.

Checked Ver<sub>0</sub> Clones - perhaps only  $\approx 5-10$  clones will survive - will need to step up efforts in future.

prepared a large batch of solutions in preparation for viral RNA production.

1M Tris pH 7.5 - Autoclave  
 1M NaAcetate pH 4.5 - Autoclave.  
 20% SDS (Not autoclaved)  
 Glycerol - autoclave  
 1M MgCl<sub>2</sub> autoclave  
 1M CaCl<sub>2</sub> autoclave  
 0.5M EDTA pH 8.0 autoclave  
 X 10 TE buffer pH 7.6  
 X 10 NTE buffer pH 7.6

C.p.e. progressing well on ATCC infected cells. p<sub>2</sub>  $\rightarrow$  p<sub>3</sub> Ver<sub>0</sub> at  $\frac{1}{2}$  dilution and p<sub>3</sub>  $\rightarrow$  p<sub>4</sub> dil<sup>n</sup>  $10^{-1}$ /undil.  
 Will probably be able to harvest this afternoon

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date



Recorded by

David K. Clarke

000071

TITLE: \_\_\_\_\_

Project No. \_\_\_\_\_

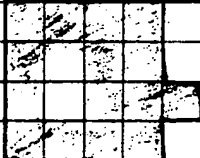
Book No. \_\_\_\_\_

49

From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub>s - final reading - titres are low

-1 -2 -3 -4 -5 -6 -7



ATCC phages

 $10^{4.75}$ 

DI build up may be a problem

-1 -2 -3 -4 -5 -6 -7 -8

 $10^5$ 18537 P<sub>2</sub> Vero 'New Cells' - Perhaps should have infected with  $10^{-1}$  dil<sup>n</sup> (DI build up may be a problem once the titer goes over  $10^5$ )

Checked Vero cell clones - there appears to be ~ 9-10 clones growing slowly

Checked c.p.e. on ATCC infected cells - they are progressing very well - will harvest ~ 4pm

prepared Microsomeal nucleic stock at  $10\text{mg ml}^{-1}$  - stored frozen (in deionised H<sub>2</sub>O)prepared DNase I  $25\text{mg ml}^{-1}$  in 5mM NaAc, 1mM CaCl<sub>2</sub>, 50% glycerol - stored -20°Cprepared Proteinase K at  $10\text{mg ml}^{-1}$  in 10mM CaCl<sub>2</sub>By Spm the c.p.e. on P<sub>2</sub> Vero and P<sub>1</sub> Vero ATCC infected cells was very extensive - decided to harvest them all even though  $10^{-1}$  P<sub>1</sub> Vero was not as extensive in c.p.e. (stored all in "working stock box" at -80°C)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sey Kishi

Recorded by

David K Clarke

000072

From Page No. \_\_\_\_\_

Carried out TCID<sub>50</sub> for P<sub>1</sub> Vero (ATCC) Dil/untl - as a test for roller bottle infection with P<sub>3</sub> stock

Also did TCID<sub>50</sub> for ATCC P<sub>3</sub> Vero - To be used as working stock for infection of Roller Bottles

Moved Nucleases + Potassium-K to 'General Freezer'

\* Vero Cloning

Kim recommended using 1ml of growth medium for 24 well plates and 2ml for 6 well plates - Rinse briefly with some PBS - Trypsinize by pipetting up and down with a yellow tip

~~Scrub~~ Lunch with Frank.

Went over protocols with Kathy - Centrifuges, tubes, volumes, speeds etc

Checked cloned Vero cells, a few more clones are showing signs of life. Where cells are growing they have a very

Checked Temp/CO<sub>2</sub>/H<sub>2</sub>O in Flu/RSV T.C. Room.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Sege Kibbi*

Date

Invented by

Recorded by

*David R. Clarke*

Date

0000073

TITLE: \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

51

From Page No. \_\_\_\_\_

9am Flu/RSV group scientific meeting

12-1pm Infected 20 Roller bottles with RSV A2 ATCC  $\beta$  Vero - Diluted  $\frac{1}{10}$  in MEM  
Used 10ul of this solution to infect each roller bottle

Adsorbed virus for  $\approx$  3hrs at 37°C in the roller cabinetThen added 45ml of MEM  $\rightarrow$  37°C for 48hrs - 72hrs

George provided me with a bottle of Ultra pure formaldehyde - stored in freezer in my fridge  
Lab. - will deionize before use and refreeze at -80°C. Need to order mixed-bed resin

Proportions for MOPS Gel.

Per 50ml

5ml x10 MOPS buffer

35ml H<sub>2</sub>O

9ml formaldehyde

Check vs. Yung's protocol.

Melt agar in H<sub>2</sub>O, cool, then add x10 MOPS and formaldehyde - cool further and pour

Checked TCID<sub>50</sub> on ATCC  $\beta$  Vero working stock and P<sub>1</sub> virus 10<sup>-1</sup>/unit.

These are the beginnings of cpe. - will check again on Monday.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Suzanne K. Kille

Date

Invented by

Date

Recorded by

David R. Clarke

0000074



From Page No. \_\_\_\_\_

Checked infected Roller bottles: There has been considerable c.p.e. caused by the virus (RSV A2 ATCC pig virus diluted  $10^{-1}$  - 1000 / Flask). Many dead cells are floating along with clumped dead cells and syncytia. Some syncytia are also visible on the remaining cells. There are sufficient remaining cells to warrant leaving until tomorrow. Hopefully by then the majority of cells will have been infected and will be free floating.

Also checked TCID<sub>50</sub>s for ATCC Pig Virus.

and ATCC Pig Virus tit/undil.

They are showing good c.p.e. out to  $10^{-4}$  - will make a final reading on 27<sup>th</sup> (wed).

Witnessed &amp; Understood by me,

Date

Invented by

Date

To Page No. \_\_\_\_\_

*Sege Kbl*

Recorded by

*David K Clarke*

000075

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub> - looks as though there is c.p.e. out to  $10^{-5}$  with  
 ATCC p<sub>2</sub> virus (working stock) but only to  $10^{-4}$  with p<sub>1</sub> virus  $10^{-1}$ /unit.  
 Will make final readings on Wed.

Assuming  $10^{5.5}$  ml<sup>-1</sup> for ATCC p<sub>2</sub> virus may need to dilute by  $10^{-2}$  and p<sub>1</sub> have

$5 \times 10^3$  p.f.u. ml<sup>-1</sup> (This should eliminate majority of D.I.s) then put 200  $\mu$ l on  
 a 96 well plate x 5. Also will try vaccination in a controlled experiment.

Checked virus clones - they are doing fine and showing signs of growth - will change  
 medium again on Thurs or Fri.

Checked all for passage (Virus left for me by Kim) - Will split tomorrow  
 into 2 x 25 cm<sup>2</sup> flasks for use on wed; a 96 well plate for this p.f.u. + a  
 TCID<sub>50</sub> for this to be passaged for the following week.

Harvested virus from 20 roller bottles - Spun at 2K rpm for 30 mins for hard spin  
 then freeze - thawed pellets each in 500  $\mu$ l MEM, repelleted for 5 mins at 2K rpm  
 pooled the supernats and then put on 0.1 spin. Retained 1.5 ml of super for TCID<sub>50</sub>.

Ordered mixed bed resin from BioRad 500g \$110.

Set up TCID<sub>50</sub> on roller bottle harvest

Put on 0.1 centrifuge spin 10K RPM 4°C in the '4' Rotor (Beckman  
 JA14) Rotor.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Sey Kble

Date

Invented by

Date

Recorded by

Dwain R Clarke

0000076

From Page No. \_\_\_\_\_

Took off virus-pelleting spin - there were substantial pellets visible in each centrifuge bottle - Decanted supernatants, drained and added 1.0 ml of nuclease compatible buffer onto each pellet. (Buffer contained 50mM Tris pH 7.5, 10mM  $MgCl_2$ , 5mM  $CaCl_2$ , 50mM Tris pH 7.5, 100mM  $MgCl_2$ , 50mM  $CaCl_2$  + 9.35 ml  $H_2O$ )

Left pellets to resuspend on ice in the cold room.

Did some cell culture - Trypsinized Vero cells from TC 150  $cm^2$  into 1ml of Tryp-EDTA. Used 200  $\mu$ l to set up a fresh TC 150  $cm^2$  Flask. Also used 200  $\mu$ l  $\rightarrow$  8ml MEM for 2 x 25  $cm^2$  flasks. Diluted 100  $\mu$ l into 10ml MEM, then diluted 2ml of that into 18ml of MEM and plated out 20  $\mu$ l/well of a 96 well plate. Cells look a little dilute, but they should be ready by mon at the latest.

Prepared a batch of 20x SSC - 3 litres - stored in mol. Biol. Lab.

Checked TCID<sub>50</sub>s - will make some final readings tomorrow

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,



Date

Invented by

Date

Recorded by



000077

TITLE: \_\_\_\_\_

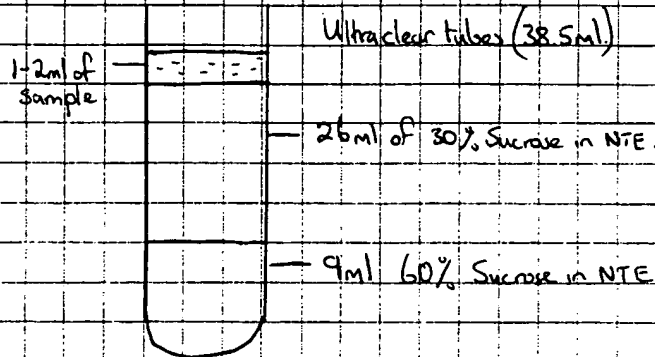
From Page No. \_\_\_\_\_

Checked Virus/ad debris pellets - resuspended them by gentle pipetting (1ml tip) - Transferred to eppendorf tubes x 5. Treated one tube <sup>(A)</sup> with

DNase I 3  $\mu$ l (50  $\mu$ l of stock)  
 Micrococcal Nuclease (100  $\mu$ l of stock)  
 RNase A (10  $\mu$ l of 10mg/ml) actually used 50  $\mu$ l  
 stock

9-45 - 10-45 am

Prepared sucrose gradients for banding of Virus



Centrifuged at 27K  
 for 45 min at 4°C  
 SW 28 rotor

21: 10<sup>5.75</sup>

ATCC p3 Vero (Used as seed for working stock)

To Page No. 56

Witnessed &amp; Understood by me,

Sey Kishi

Date

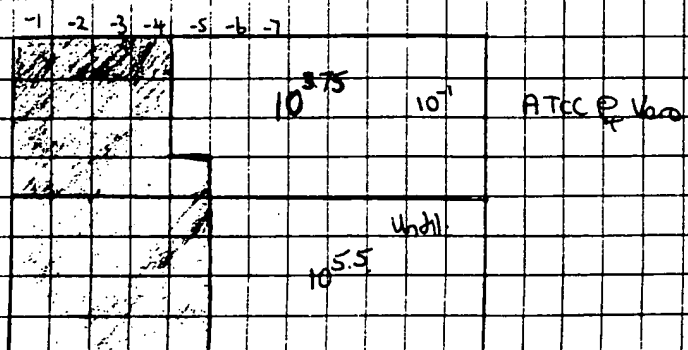
Invented by

Date

Recorded by

David A. Clarke

0000078

From Page No. 55

Checked cell monolayers which I set up yesterday.

The 25cm<sup>2</sup> Flasks will be ready tomorrow.  
the TC 150 will be ready for passage on Fri - the Abwell plates should also be ready by Fri.

After Nuclease treatment of Sample (A) diluted in NIE and repelleted along with the untreated (B) sample. (NIB let a bit of cell debris settle out of both samples - only pelleted the material above the settled debris.)

After running sucrose gradient saw a large milky band at the interface of the 30% sucrose and 60% sucrose along with much cellular debris. Harvested off the bands - mixed with STE and repelleted along with (A) + (B) above in the SW 28 (38.5ml bucket) 3-4.5 spin in. 1 hr 40 mins at 27K + °C.

(It does not seem at the moment that the sucrose gradient has been of much use in the purification scheme, but we must wait and see.)

Decanted supernatants from pellets, and resuspended (O/N on ice) A and B in STE x1 - resuspended C and D in nuclease compatible buffer. Used 1ml for resuspension.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Sege Kobi

Date

Invented by

Date

Recorded by

David K. Clarke.

0000079

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

Resuspended 'virus pellets' in either STE or nuclease compatible buffer  
(A) + (B) in STE C + D in nuclease compatible buffer. Added 50 $\mu$ l of  
Stock DNase I + 50 $\mu$ l of stock Micrococcal nuclease + 1 $\mu$ l RNase A - mixed and  
incubated at 37°C for 1 hr (9am - 10am)

(Inhibited Micrococcal nuclease with EDTA)

Following Nuclease treatment will set up proteinase K reaction in X1NTE, 0.2%  
SDS and 1mg/ml proteinase K. - 1 hr at 37°C

Checked Vero clones - not a large amount of growth - will remove some old  
medium and add some fresh medium this afternoon.

Preparing Proteinase K in  $\text{CaCl}_2$  may have been a mistake since the  $\text{CaCl}_2$  may  
supply enough  $\text{Ca}^{2+}$  for micrococcal nuclease - although there should be enough EDTA  
to take care of it.

Infected 2 x 25cm<sup>2</sup> Flasks each with 200 $\mu$ l of ATCC p3 Vero - adsorbed for 2-3 hrs

Replaced some of the growth medium on the Vero clones with fresh growth medium

Witnessed &amp; Understood by me, \_\_\_\_\_

Serge Kibbe

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

David K Clarke

To Page No. \_\_\_\_\_

000080

From Page No. \_\_\_\_\_

Spin down 1ml from each of genomic RNA preps - dried down pellets  
(looked a little too large and white to be RNA - they are probably DNA)

Prepared 10M NaOH, 1M NaCl, 1% methylene blue, 1M NaAc pH 5.2 in preparation  
for Northern blot work.

Resuspended my pellets in 10µl x1 mops gel loading buffer, 10µl ultra pure formamide  
and heated to boil for 5-10 mins. Then added 5µl of Fw/BBB and loaded  
gel.

L.H.S

/5µg Plasmid DNA / 0.2µl (1µg) buffer / 2µl (2µg) buffer / A / B / C / D /

Run gel for at 80mA until BBP was at the bottom of the gel.

Need to check infections (from yesterday), 96well plates; set up TC150 for mon/tues.

Infection is proceeding well, will harvest tomorrow afternoon + sonicate +/-

Set up a TC150 for monday using 200µl out of (1000µl of trypsinised cells)

Proceeded with N.Blot after the BBP had run to the end of the gel. - Soaked in  
3 changes of H<sub>2</sub>O (each for 5mins - to remove form) then soaked in 50mM NaOH 10mM  
NaCl for 15mins then for 2 hrs in 100mM Tris pH 7.5 (this could be shortened), then  
for 30mins in 20x SSC - Then set up blot.

Will harvest tomorrow and UV cross-link on RNA. Will wait  
until Monday to strain the filter.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge Kibiki

Recorded by

David K Clarke

0000081

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Dismantled N. Blot (from yesterday) - allowed filter to air dry - it was still damp, and then u.v. cross-linked X2 using the settings George showed me. Stored in my Lab Drawer at R.Temp.

Checked infected Vero - C.P.R. was v. extensive - harvested both 25cm<sup>2</sup> flasks.

Sonicated one harvest (3x 2secs at setting Frances uses) - sonication was very powerful and frothed the medium and disintegrated the cell debris.

Freeze-thawed the other harvest - then carried out a TCID<sub>50</sub> to compare phn from each harvest technique.

Tidied T.C. room and restocked (final week of responsibility!)

Witnessed &amp; Understood by me,

*Sege Kibiki*

Date

Invented by

Date

Recorded by

*David K. Clarke*

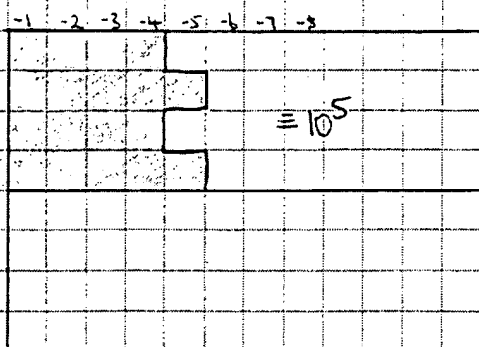
To Page No. \_\_\_\_\_

0000082



From Page No. \_\_\_\_\_

Did final Read on TCID<sub>50</sub> for Virus harvest from 20 Roller bottles



BSA 2 ATCC p<sub>+</sub> Vero (Roller bottle harvest)

It's possible that there were many more particles produced, but they lost infectivity

Checked Cloned Vero cells. There are at least 3-4 Vero clones which are growing quite well, will check again on Wed and will refed and/or

subclone cells into 24 well plates

Carried out staining of N. Blot filter

15 mins in 5% Acetic Acid

10 mins in 0.04% Methylene Blue

2-3 mins in H<sub>2</sub>O

The larger fragments on the RNA marker lanes did not transfer efficiently to the filter, which may explain the absence of any visible genomic RNA. There should be  $\approx 250$  ng/band in the marker lane

Although this did not look encouraging, decided to proceed with Oligo synthesis for cDNA/PCR during

RNA markers

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sage Kelli*

Recorded by

*Warren K. Carter*

0000083

TITLE: \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

61

From Page No. 60

Passaged Vero cells (2x10<sup>6</sup> from 1ml of hypinized cells) into a fresh TC150

Decided to order 2 oligos for cDNA synthesis/PCR - located in the L-gene - at position #12245 and position #12775.

①

- Primer for cDNA synthesis

5'-GTTTAAACACGTGGTGAG-3'

Called - PmLI → Hph I.

⊕ sense will be 1<sup>st</sup> primer - use for cDNA synthesis.

SDc m.p. for both primers

11mers + 18mer

②

5'-ATCAACATCACGTGTGA-3'

Called - Hph I → PmLI.

⊖ sense - will be back primer.

Received Oligos 11-3-93.

\* NB paper on 'N' gene sequence of BRSV. Virology 180, p453-456 (1991) Samal et al.

Need to check Ovine RSV paper (at home).

Dave gave me a print-out of the RSV-CAT constructs.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve K. White*

Recorded by

*David R. Clarke*

000084

From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub> on Sonicated/Freeze Thawed comparison At the moment it looks as though both are giving the same titer ( $\approx 10^5$  pfu/ml) - So much for sonication

Contacted J. Beeler (on maternity leave) for RSV Subgroup B Mac/9320/77 (She will be back in early Dec)

Checked references on preparation of nucleocapsid for PCV3 and RSV - they are essentially the same as the procedure used in John's lab - need to check in methods notebook

Ordered 2<sup>nd</sup> oligo for cDNA/PCR RSV (called it "HptMT back to PstI")

Planned out Oligos needed for making synthetic CAT/RSV construct

Need oligos to PCR out CAT gene from plasmid, then need <sup>(eight)</sup><sub>(six)</sub> more oligos to synthesize the

① Xba-CAT Oligo 5'-TAACTCTAGAATGGGAGAGAAAT 24-mer

4 extra nucleotides to extend past XbaI site for cutting purposes

ordered

② Pst-CAT Oligo 5'-AATACCTGCAGTTACGCCCC 19-mer

first cloning PstI site  
next hand out PstI cut site

ED. Moczarski

723-6435

⊗ New concept - a totally or partially synthetic RSV genome

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge Khril

Recorded by

David K. Clark

000085

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub> for freeze-thaw/sonicated comparison of titers.  
 Titer appears to be identical  $\approx 10^{5.25}$  - Final reading tomorrow or Fri.

Checked Vero clones. There are 5-6 Vero clones showing fairly good growth.  
 I will expand 2 of these clones into a 24 well plate, and will feed the remainder.

Need to Order Oligos for RSV ends (to be added to the cat gene)

① 96-mer 'Kpn to Xba RSV Plus'

5'-CGACGCGATATTACGCCGAA AAAATGCGTACAACAAC TTGCAT AAA  
 CCA AAA AAA TGG GGC AAA TAA GAA TTT GAT AAG TAC CAC TTA  
 AAT TTA ACT-3'

② 104-mer "Xba to Kpn RSV minus"

5'-CTAGAGTTA AATTAA AGT GGT ACT TAT CAA ATT CTT ATT TGC  
 CCC ATT TTT TTG GTT TAT GCA AGT TTG TTG TAC GCA TTT TTT  
 CGC GTA ATA TGC GTC GGT AC -3'

① 50mer "Rse I to Hind III: A, RSV minus"

5'-GTATTCAAT TAT AGT TAT TAA BAA TTA AAA ATC ATA TAA  
 TTT TTT AAA TA -3'

② 60mer "Rse I to Hind III: B, RSV minus"

5'-ACT TTT AGT GAA CTA ATC CTA AAG TTA TGA TTT TAA TCT TGG  
 AAG AAT AAA TTT AAA CCC -3'

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,




Date

Invented by

Date

Recorded by



0000086

From Page No. 63

③ 86-mer "RFL to Hind III : C, RSV Plus"

5' TAA TCT AAT TGG TTT ATA TGT GTA TTA ACT AAA TTA CGA GAT ATT  
 AGT TTT TGA CAC TTT TTT TCT CGT TAT AGT GAG TCG TAT TA

↑  
 one correction

④ 56-mer "Hind III to RFL: ~~B~~ RSV minus"

5' - AGC TTA ATA CGA CTC ACT ATA ACC AGA AAA AAA GTG TCA  
 AAA ACT AAT ATC TCG TA -3'

⑤ 60-mer "Hind III to RFL: Y RSV minus"

5' ATT TAG TTA ATA CAC ATA TAA ACC AAT TAG ATT AGG GTT TAA  
 ATT TAT TCG TCG AAC ATT -3'

⑥ 88-mer "Hind III to RFL X RSV minus"

5' - AAA ATG ATA ACT TTA GGA TTA GTT CAC TAA AAG TTA TTT AAA  
 AAA TTA TAT GAT TTT TAA TTT TTA ATA ACT ATA ATT GAA TAC TGC  
 A -3'

Tai An delivered Oligos for cDNA synthesis/PCR - stored at -80°C in H<sub>2</sub>O.  
 a 1/10 to 1/20 dilution will give 100 ng  $\mu$ l conc

Expanded 2 Vero clones which were growing well - trypsinized them in  
 50  $\mu$ l of Tryp-EDTA after 2 mixes with PBS - pipetted into 100  $\mu$ l of MEM and  
 transferred to a 24 well plate - then added fresh growth medium.  
 Fed remainder of Vero clones with fresh growth medium.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge Kibbi

Recorded by

Harold K. Clatter

000087

TITLE: \_\_\_\_\_

From Page No. 64Will use 1  $\mu$ g of CAT Plu construct for PCR of CAT gene

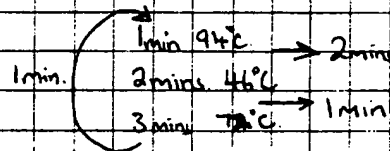
Conditions for

CAT amplification.

1  $\mu$ l (1  $\mu$ g) of plasmid DNA - get from Dave.1  $\mu$ l of each primer (working pair)2  $\mu$ l 10mM dNTP5  $\mu$ l  $\times 10$  RXN buffer38  $\mu$ l  $H_2O$ 1  $\mu$ l Enz after 2 min' burn'  $\rightarrow$  Ice  $\rightarrow$  Spin  $\rightarrow$  Rtemp.Will move from 72°C  $\rightarrow$  94°C as quickly as possible RAMP 4

Will move slowly from 94°C to annealing temp RAMP 2/3

Will move from 72°C (annealing temp) to 72°C rxn temp at RAMP 3



programmed PCR

Double checked Oligo sequences.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Seize Kibbi*

Date

Invented by

Date

Recorded by

*David K. Clarke*

0000088

From Page No. \_\_\_\_\_

9am Performance rating meeting

Prepared 10mM Methylmercury and 1.85M 2-Mercaptoethanol

Spun down 2.5ml of Genomic RNA preps A and B, 70% ethanol washed and dried - held at -10°C until ready for cDNA synthesis

Will prepare 200ng/μl working stocks of primer in H<sub>2</sub>O

Set up cDNA synthesis exactly according to the protocol in Lab methods book. Used 2 μM of reverse transcriptase

Allowed reverse transcription to proceed - then inactivated at 95°C for 5 mins. At this point one of the tubes popped open and some water both water was taken into the reaction mixture - however proceeded with both samples for PCR - worked with the mineral oil (beside the PCR machine). Ran program N° 28 (see page 65 for conditions)

Need to start Hep-2 cells growing, to see if higher titers can be attained in these cells vs Vero.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Serge Kibiki*

Recorded by

*David K. Clarke*

0000089

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

Took off PCR mixtures - held on ice until ready to run gel

Prepared a 12 Agarose gel - loaded samples

LHS. 1Kb Ladder / Space / (1) Nuclease / (2) Nuclease /  
41pg

loaded 10µl of PCR rxn mixture which contained some 'gunk' at the mineral oil/H<sub>2</sub>O interface.

1Kb Ladder

5090  
1072  
3054

2036  
1636

1018

(MAB-13-1 is on its way from Bruce Ferrie and hybridoma cells will be available later). It reacts to an epitope in Antigenic site B. It does not neutralise the Aus/92/61 strain, but will neutralise Mass/9320/77 (Subgroup B virus)

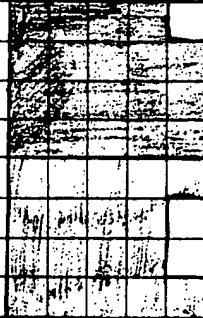
cDNA/PCR rxn did not work. Should probably run a

Flu cDNA synthesis reaction control.

Did final read on TCID<sub>50</sub> for Sonicated vs Freeze-Thaw

506/517  
396

-1 -2 -3 -4 -5 -6



Sonicated

Freeze-Thaw

Not much difference!

Will culture Vero into a fresh TC150 X2 - will do an exp on Monday to assay virus production in a timed manner

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzi Kelle

Recorded by

David K. Clatke

UUUUU90



From Page No. 67

Passed Vero cells into 2 x 160 cm<sup>2</sup> bottles - will infect one bottle on Monday with  
1ml of working stock virus

Checked 2 subcultured Vero clones - they are doing fine - will check again on Monday

⊗ B-subgroup Virus will soon be on its way

Will ask Kim to break out Hep-2 cells and adjust one of the incubators to 32.5°C

Witnessed &amp; Understood by me,

Date

Invented by

Date

To Page No. \_\_\_\_\_

*Sign W. K. K.*

Recorded by

*David K. Clarke*

000091

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Prepared stock x10 Analyte electrophoresis buffer and 50% glycerol BPE in H<sub>2</sub>O

Redesigned Oligos for Tail Am - into 450mers

for the leader Region

KpnI ① 46mer ② 50mer XbaI

3mer ③ 50mer ④ 24mer

① 5' CGA CGC ATA TTA CGC GAA AAA ATG CGT ACA ACA AAC TTG CAT  
46mer. ✓ AAC ordered

50mer ② 5' CAA AAA AAT GGG GCA AAT AAG AAT TTG ATA AGT ACC ACT TAA ATT  
✓ TAA CT ordered

24mer ③ 5' CTA GAG TTA AAT TTA AGT GGT ACT ~~TAT CAA AT~~ ordered

50mer ④ 5' TAT CAA ATT CTT ATT TGC CCC ATT TTT TTG GTT TAT GCA  
✓ AGT TTG TTG TA ordered

30mer ⑤ 5' CGC ATT TTT TCG CGT AAT ATG CGT CGG TAC  
ordered

Oligos also have to be designed for the trailer region.

P.T.O

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Serge W. Khle*

Recorded by

*David & Clarke*

000092

From Page No. 69

Trailer Oligos.

①

50mer

②

50mer

③

50mer

④

40mer

29mer

⑨

50mer

⑧

50mer

⑦

50mer

⑥

25mer

⑤

✓ ① 5' GTA TTC AAT TAT AGT TAT TAA AAA TTA AAA ATC ATA TAA TTT TTT  
50mer AAA TA ordered

✓ ② 5' ACT TTT AGT GAA CTA ATC CTA AAG TTA TCATTC TAA TCT TGG AGG  
50mer AAT AA ordered

✓ ③ 5' ATT TAA ACC CTA ATC TAA TTG GTT TAT ATG TGT ATT AAC TAA  
50mer ATT ACC AG ordered

✓ ④ 5' ATA TTA GTT TTT GAC ACT TTT TTT CTC GTT ATA GTG AGT CGT  
40mer ATT A ordered

✓ ⑤ 5' AGC TTA ATA CGA CTC ACT ATA ACG A  
25mer ordered

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge W. Kuhl

Recorded by

David K. Clarke

000093

TITLE: \_\_\_\_\_

From Page No. 70

✓ ⑥ 5' GAA AAA AAG TGT CAA AAA CTA ATA TCT CGT AAT TTA GTT  
 Smir AAT ACA CATAT ordered

✓ ⑦ 5' AAA CCA ATT AGA TTA GGG TTT AAA TTT ATT CCT CCA AGA TTA  
 Smir AAA TGA TAG ordered

RSV-LAT leader-7  
 Should actually be trailer

✓ ⑧ 5' ACT TTA GGA TTA GTT CAG TAA AAG TTA TTT GAA AAA TTA TAT  
 Smir GAT TTT TAA ordered

✓ ⑨ 5' ATT TTT AAT AAC TAT AAT TGA ATA CTG CA  
 29 mer. ordered

If RSV infectivity is largely associated with cellular debris, then can use that  
 hint to concentrate the virus - however may need to plaque pick and/or go to  
 Hep-2 cells

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Steve W. Kuhl

Recorded by

David K. Clarke

000094

From Page No. \_\_\_\_\_

Checked Vero Cells - will pass and infect today - Infect at 10am with  
 P<sub>2</sub>Vero (working stock - 1ml) - will remove inoculum after 3hr adsorption.

Vero clones are doing fine - will sub-culture 3 more today - and feed the others  
 tomorrow.

10am Initiated infection using 1ml of working stock p<sub>2</sub>Vero on the TC 50  
 adsorbed 3hrs - then added 20ml of MEM and returned at 37°C.

Ⓢ Asked Kim for Hep-2 cells. Adjusted one incubator to 32.5°C for temp. exp.

Set up 2 x 25cm Flasks for Wed to carry out time course experiment - will  
 harvest at 24, 30, and 48h p.i. (2 x 1.5ml) - will leave 1ml aliquot untreated - will spin the  
 other in the microfuge and assay the supernatant for infectivity. (will try to resuspend pellets  
 in 100µl MEM and assay that)

Ⓢ Will need 4 x 96 well plates for Fri

Sub-cultured 3 more Vero clones into 24-well plates

Will ask Ying for 'Cat plasmid' and book for machine as soon Tai An delivers the oligos

Checked all oligos needed for production of rsv-ent for CAT work will order this  
 afternoon.

Ordered 100g of Na<sub>2</sub>Deoxycholate from Sigma - should arrive by week's end

Prepared 1 litre of 10x Versene solution and 1 litre of 10x Hepes/NaCl/MgCl<sub>2</sub>  
 for hypotonic resuspension buffer

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Suzanne K. Kuhl

Date

Invented by

Date

Recorded by

David R. Clarke

000095

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

Checked infected cells - will start harvest at 10am - there ~~are~~ is already a pronounced c.p.e. but not many cells floating in the medium.

\* NB Density of Ncapsid  $1.31 - 1.32 \text{ g cm}^{-3}$

Estimate 100s for Ncapsid S-Value (not necessary)

Was able to calculate time run length for SW28 at 25K to give equivalent sedimentation value as SW41 spin.

Spin length/conditions are 3hrs 50mins at 25K,  $12^\circ\text{C}$  in SW28

for cell harvest (see above) Collected 3ml into 50ul Hapes/Sol.  $\text{MgSO}_4$  - mixed and aliquoted 1.2ml into each of 2 eppy tubes. Spun one tube at  $4^\circ\text{C}$  10K xg for 5mins (did nothing to the other) Harvested Super from spin tube and froze both samples in an EtOH/dry ice bath.

12pm put on restriction digest Hind III on CAT plasmid given to me by Ying.

5ul x 10 buffer

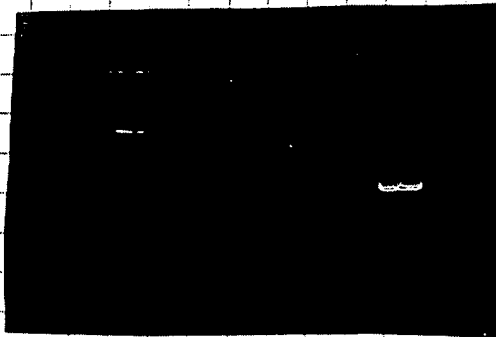
2ul plasmid ( $\approx 9\mu\text{g}$ )

45ul  $\text{H}_2\text{O}$

50ul + 1ul Hind III ( $20\text{u}\mu\text{l} = 1$ )

Analyse: 2ul on a 1% Agarose gel with ladder to check it was cut.  
(Ruth ran the gel)

Hind III cut CAT  $\approx 4\text{kb}$   
Plasmid  
(Fh).



Uncloned plasmid may pose a background problem, will EL EL PCR product

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sign W. K. L.*

Recorded by

*David K. Clarke*

0000096

From Page No. 73

~~1/2~~ Completed 30hr harvest from RSV pg. vero infected cells - spun one appendage  
and left the other as is. Freeze both and stored at -80°C.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Koble*

Recorded by

*David K. Clarke*

000097-

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

Sent fax to John with Paper Corrections etc.

10am - Carried out harvest of virus at 48h p.i. - spun one sample as before and retained supernatant - froze down both aliquots. Also scraped monolayer (which was showing complete fusion but not completely disintegrating) into remaining MEM (20ml) - Pelleted - 4°C 3K rpm 10 min - poured off super and resuspended pellet in 1ml MEM/100µg/ml myo - freeze - thawed - respun at 1.5K for 5 mins and collected the super - froze at -80°C

Checked Vero clones - 2 more clones will be ready for sub-culture tomorrow

Will feed 2 of the clones in the 24 well plates (and pass them on then or <sup>Fr</sup>)

prepared another series of RXN buffers for Nucleoside preparation.

X10 Tris/NaCl/EDTA pH 7.5

10% SLS

40% Sucrose

programmed PCR machine for Rescue of CAT gene from Flu construct.

1min at 94°C

2mins to cool to annealing temp

2mins at 49°C Annealing temp

1min to heat to RXN temp 72°C

3mins at 72°C

1min to get back up to 94°C

Infected 2 x 25cm<sup>2</sup> Flasks - each with 30µl of RSV p3 Vero - adsorbed one at 37°C for 2-3hrs and the other at 32°C for 2-3hrs

Set up PCR RXN - collected Hind III cut CAT plasmid

70µl EtOH washed - Dried

Resuspended in 7µl H<sub>2</sub>O

Used for PCR RXN

Ordered G-protein from VWR

See Overpage

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sgt W. Kille

Recorded by

David K Clarke

000098



From Page No. 75

4  $\mu$ l (1  $\mu$ g) DNA  
1.5  $\mu$ l Each primer (100  $\mu$ g  $\mu$ l<sup>-1</sup>)  
2  $\mu$ l dNTPs (10 mM)  
5  $\mu$ l x10 Rxn buffer  
38  $\mu$ l H<sub>2</sub>O

49  $\mu$ l - Heated at boiling for 2 min snap cooled spun briefly added

1  $\mu$ l of Taq pol. overlaid with mineral oil and started  
PCR Rxn.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Sage W. Kibbi*

Date

Invented by

Date

Recorded by

*David K Clarke*

000099

From Page No. \_\_\_\_\_

Took off PCR Rxn, prepared 1% Agarose gel and analysed 5 $\mu$ l of PCR products alongside DNA 1 Kb ladder.

IF Rxn has worked will <sup>Phenol</sup> chloroform extract, EtOH ppt, digest with Xba I / and Bst I gel purify the fragment.

Oligos need to be gel purified and kinased (except for those with free 5' ends).

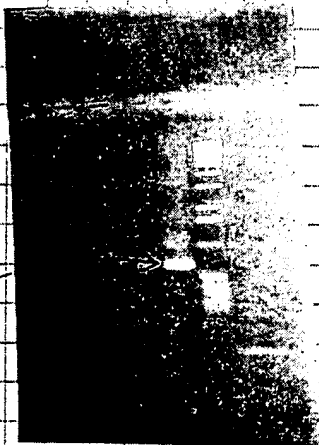
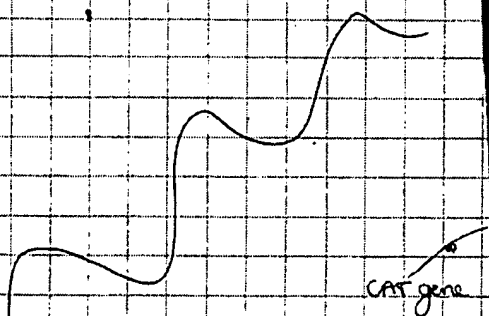
Cheered infected cells 32°C/37°C - No significant differences in c.p.e. - although both monolayers do look infected.

Ordered Phenol/chloroform/isoamyl pH 6.7 and Chloroform/isoamyl  
25 : 24 : 1 and 24 : 1

Prepared a 60% w/v soln. of CsCl in H<sub>2</sub>O - Density = 1.46 g.mL<sup>-1</sup>

When making CsCl gradient will put  $\frac{1}{2}$  of the 40% layer as 60% to ensure N capsid doesn't pellet through.

Photographed amplified CAT gene - looks pretty good  $\approx$  1-2  $\mu$ g total CAT gene DNA (size  $\approx$  700 b.p)



Completed  $\phi$  chloroform extraction and EtOH ppt<sup>ed</sup> on at -10°C.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Sage W. Kibbe*

Date

Invented by

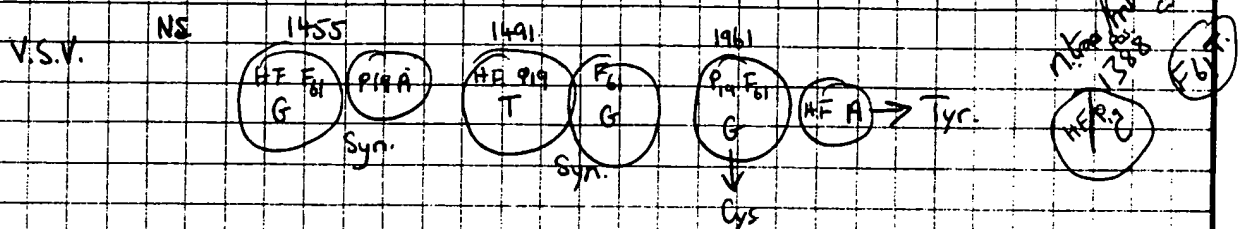
Date

Recorded by

*David K. Clarke*

000100

Checked infectivity at 32°C and 37°C. - C.p.e. is v. advanced at 37°C but not so advanced at 32°C



Subcultured ~~the~~ Vero clones ① and ② into 6 well plates in duplicate, also subcultured one more clone from initial 96 well plate into a 24 well plate (however only a few cells made it through the transfer - probably because the cells had been growing for a long time and were not firmly fixed to the plastic.)

Will carry out XbaI digestion at 37°C for 1-2hrs in 20µl - followed by PstI digestion at 37°C in 50µl.

Set up Xba I digest.  
2  $\mu$ l Xba buffer.  
1  $\mu$ l BSA (1000  $\mu$ g/ml)  
1  $\mu$ l  $\text{H}_2\text{O}$   
1  $\mu$ l Enz  
20  $\mu$ l 37°C 1.5hr

Then added 5  $\mu$ l x10 Pst I rxn buffer + 2  $\mu$ l  $H_2O$  and incubated at 37°C for  
a further 2 hrs.

Meanwhile, set up 4 TCID<sub>50</sub>s to compare virus titers for P<sub>3</sub> Vero 24hr, 30hr, 48hr harvests - spun/not spun and for freeze-thawed cellular material X10 conc after 48hr

**To Page No.\_\_\_\_\_**

**Witnessed & Understood by me.**

**Date****Invented by**

Date \_\_\_\_\_

Aug W. Koble

Recorded by

Recorded by David K Clarke

000101

TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

79

From Page No. 78

Harvested virus from 32°C/37°C infections. Cell monolayer at 37°C was blown away (Tah. pip.). The cell monolayer at 32°C was completely syncytiated but was not falling apart as at 37°C.

Resuspended cell debris pellets in 1ml of MEM.

Froze down 3 x 1ml aliquots from each harvest.

Checked conc. of NP-40 supplied (in stockroom).

Made up 10% Na Deoxycholate (100ml).

Also prepared 5M NaCl (500ml).

1M Tris (1L) pH 8.0.

Need to call Sigma for N-P 40 conc.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Age W. Kble*

Recorded by

*David K. Clarke*

000102

From Page No. \_\_\_\_\_

Checked Vero cells to be infected with RSV for Nucleocapsid.  
Will leave them until tomorrow.

Checked TCID<sub>50</sub> - although results are preliminary it looks as though similar amounts (but v. low) of virus are released into the supernatant from infected veros at 24<sup>and</sup> 30 h.p.i. These virus appears to be free-floating and not associated with cellular debris at these "early stages". At 48 h.p.i. a greater percentage of total infectivity appears to be cell associated. Also at 48 h.p.i. the majority of infectivity can be pelleted at 3000 rpm and released from the cell pellet by one cycle of freeze-thaw in 1 ml MEM ( $\approx$  100 fold more virus appears to be cell associated at 48 h.p.i. vs free floating).

Will shoot for N. capsid prep" at  $\approx$  30 h.p.i.

George prepared a low M.P. Agarose gel - will run my trimmed CAT gene

1000 g  $\approx$  2200 r.p.m. on Table top centrifuge.

Irradiated gel with long wave U.V. light (to minimise U.V. damage) - there appeared to be 1-2  $\mu$ g of DNA in the appropriate band - excised this from the gel - melted down at 65°C for 10-15 mins, added  $\frac{1}{10}$  vol (35  $\mu$ l) of 10x Agarose buffer and 2  $\mu$ l of Agarose and placed at 42°C for 1 hr. Then added  $\frac{1}{10}$  vol. 3M Na Acetate, mixed and held on ice for 15 mins, then spun for 15 mins and took supernatant, added 2  $\mu$ l of Isopropanol - held on ice for 5-10 mins, then spun down for 10 mins - etOH (70%) rinsed pellet and dried in speed vac. <sup>fresh</sup> resuspended pellet in 50  $\mu$ l H<sub>2</sub>O and analysed 5  $\mu$ l on a 1% agarose gel.

Checked Vero clones - Will subclone N<sup>o</sup> 3 into 6 well plates tomorrow, and will infect ① and ② with RSV (may need to subculture into 25 cm<sup>2</sup> Flask)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Sege W. Kibbe*

Date

Invented by

Date

Recorded by

*Maurice K. Clarke*

TITLE \_\_\_\_\_

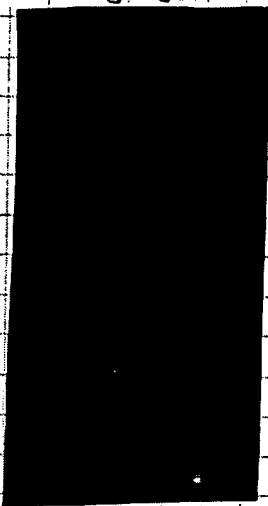
Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

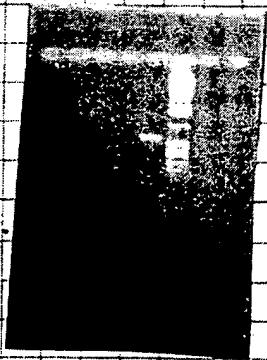
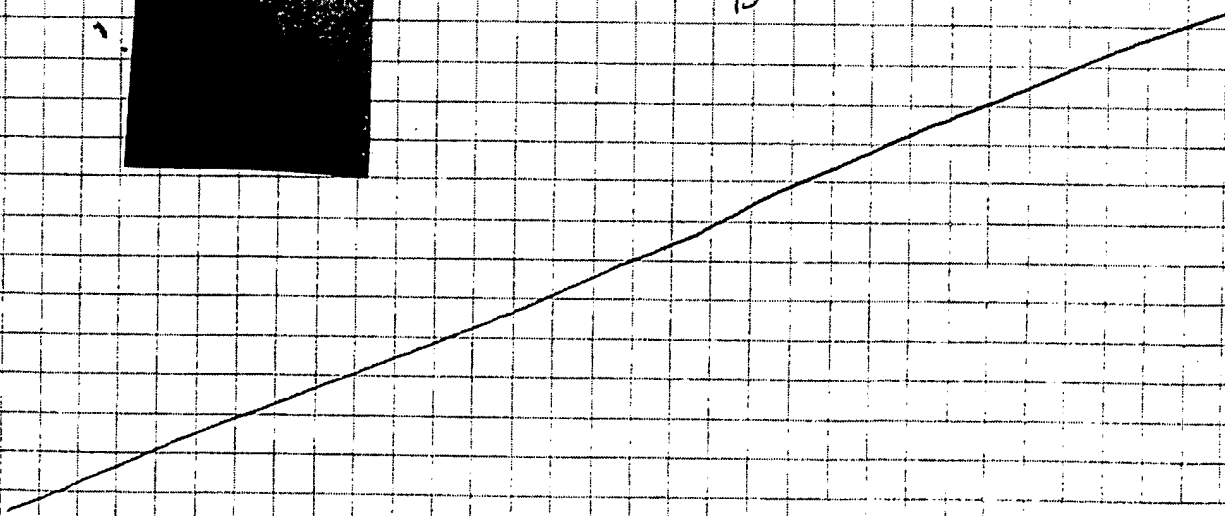
81

From Page No. 80

George's Gel



Gel analysis of Xba/BclI trimmed and gel purified cat gene.

Reran 1  $\mu$ l of purified DNA to check if the 'trimmed' DNA species is actually one DNA species as there is some doubt from this gel.Ran 1  $\mu$ l of Marker 1 Kb ladder as well.2<sup>nd</sup> gel shows that there is only one species of DNA of the correct size (the cat gene).Based on this (and the above gel) it would appear there is  $\approx 2 - 2.5 \mu$ g of this DNA.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve Kibbi*

Recorded by

*Daniel R. Clarke*

000104

From Page No. \_\_\_\_\_

See reference binder #22A

Infected 10 TC 150s, each with 1ml of P<sub>3</sub> Vero (working stock)

Absorbed at 37°C for ~3 hrs (Kept 10 bottles as control)

Also infected 1x25cm<sup>2</sup> flask of Hep-2 cells with 300µl of working stock.

Fed each bottle with ~10ml of MEM.

Checked TCID<sub>50</sub> - looks as though very few virions are budded out from the cells - even at 48h p.i. - Most virions appear to remain associated with the cellular debris and cells even when the cell monolayer is essentially wiped out.

RSV subgroup B virus arrived from Mike Hendry (Strain 9320) - stored at -80°C in Box with Judy Beele's stuff.

Subcultured Vero clone N° 3 into a 6 well plate. Subcultured clone 1

into a 25cm<sup>2</sup> flask. Also infected Vero clone ① in the 6 well plate - added 100µl of P<sub>3</sub> Vero 'Working stock' and absorbed for ~2.25 hrs at 37°C.

Then added 1-2 ml MEM and continued incubation for 48 hrs.

Ordered 250ml centrifuge bottles from Beckman.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Lyle W. Kuhl

Date

Invented by

Date

Recorded by

Daniel K. Clatter

000105

TITLE

Oligo Purification à la Tai An.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

83

From Page No. \_\_\_\_\_

Checked infected TC 150s - They are showing the beginning stages of c.p.e.  
if c.p.e. are not sufficiently advanced by 3 pm - will place at 32°C on  
↓  
to start harvest.

Checked infected Vero <sup>Vero</sup> Clone ① - No obvious c.p.e. yet - will check again at 6 pm.

Checked Vero subclones ② - they are ready for

Purified 5 leader oligos according to the protocol of Tai An.

① 5ml Methanol

② 210ml H<sub>2</sub>O

③ load Oligo (in elution buffer - got stuck) ~ 5ml

④ Reload Oligo ~ 5ml

1 drop } ⑤ Rinse with Oligo buffer 5ml

⑥ Rinse with H<sub>2</sub>O - 5ml

⑦ Elute in 3ml of 50% Methanol

Dried down in speed vac.

Placed infected TC 150s at 32°C to slow down development of c.p.e.  
will place at 37°C tomorrow morning prior to Necrotic harvest

Checked Hep-2 infected cells - a little c.p.e. is apparent - will check again  
this evening and again tomorrow morning

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sege W. Khl*

Recorded by

*David & Clarke*

0000106



From Page No. 83

Prepared XI Versene, XI Hepes/NaCl/MgCl<sub>2</sub>

40% CCl<sub>4</sub> = 24ml 60% CCl<sub>4</sub>  
 36ml x10 buffer (The etc)  
 750ul S.L.S (10%)  
 to 36ml with H<sub>2</sub>O

30% CCl<sub>4</sub> = 18ml 60% CCl<sub>4</sub>  
 36ml x10 buffer  
 750ul S.L.S  
 to 36ml with H<sub>2</sub>O

25% CCl<sub>4</sub> = 15ml 60% CCl<sub>4</sub>  
 36ml x10  
 750ul S.L.S  
 to 36ml with H<sub>2</sub>O

5% Sucrose - 5ml (40% Sucrose)  
 4ml x10 buffer (as above)  
 800ul S.L.S  
 to 4ml with H<sub>2</sub>O

Infected Vero clone (2) with 0.1ml of RSV Pg Vero (working stock). Adsorbed  $\approx$  2.5 hrs.

Also passaged duplicate Vero clone (2) into a TC 25 cm<sup>2</sup>.

Checked growth of Vero clone (1) in 25 cm<sup>2</sup> Flask.

Measured O.D.s of purified Oligos which were resuspended in 100ul sterile H<sub>2</sub>O  
 (Used Sp + 99.6 H<sub>2</sub>O) - See Oligo folder for details  
 of both. All were at least 70% total in the 100ul aliquot.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge W. Kivik

Recorded by

David K. Clarke

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

85

TITLE: \_\_\_\_\_

From Page No. 84

Checked - Vero clone ① in 25cm<sup>2</sup> flask is growing well - will probably freeze down on Friday. Clone ③ is growing well in 6well plate - will split into two 6well plates on Fri and feed 4, 5 and 6 as they are very sparse - will use fresh medium from Kim.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Lyle W. Kille*

Recorded by

*David K. Clarke*

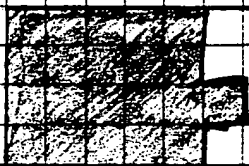
000108

From Page No. \_\_\_\_\_

Checked infected Vero clone ① - c.p.e. Now apparent - will harvest this afternoon or tomorrow - Hep-2 cells are also looking pretty sick - will leave until this afternoon or tomorrow.

Checked infected TC 150s - on 5 of flasks cpe looks very nice - lots of syncytia, on the other 5 cpe is not as extensive - placed all 10 at 37°C until 10am ± (1.25 hrs) \*

Carried out N. capsid extraction procedure.



Freeze-Thaw Soap x10 conc. 48h pi.



Spun

24h pi.

May be some contribution from added inoculum - needs to be retested after removal of inoculum

Unspun

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sege W. Kibbe*

Recorded by

*David K. Clarke*

0000109

TITLE \_\_\_\_\_

From Page No. 86

Spin

30h pi

Unspin



Spin

48h pi

Unspin

More and more virus is associated  
with cellular debris at this point  
in the infection

Harvested Virus from Vero Clone ① infection - managed to remove most of the  
cell monolayer by pipetting - added 100µl Hepes/50µl MgCl<sub>2</sub> - went through  
one freeze-thaw cycle without spinning - aliquotted and froze

Set up some bacterial cultures (Top 10 E<sup>-</sup> - nutbruges) containing PUC 19 -  
streaked colonies on Amp<sup>r</sup> Agar (used 100µg ml<sup>-1</sup> in liquid broth) -

Checked CSCI gradients after spin - Nothing was visible on either control  
or infected cell lysates.

Harvested Virus from Hep-2 cells - saved 1ml for Spin microphage spin etc.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sgt. W. K. Kelle

Recorded by

David K. Kelle

000110

From Page No. \_\_\_\_\_

Checked c.p.e on Vero clone ② infected with P<sub>3</sub> Vero (100 $\mu$ l) - V. extensive  
may indicate this Vero clone is v. susceptible to RSV.

Carried out Alkaline lysis technique for mini preps - used  $\approx$  6ml total  
volume of 96h bacterial culture.

Stored final N. acid ppt. on dried pellets at -10°C - Ran 3-4 $\mu$ l of 20 $\mu$ l  
from  $\frac{1}{4}$  (1 tube) of total prep. Loaded  $\approx$   $\frac{2}{3}$  of that sample.

There appeared to be  $\approx$  200-500 ng of DNA on the gel - so can use  
 $\frac{1}{2}$  of total prep - will purify on a low mp gel first - then cut with suitable/appropriate  
enzymes.

Took advantage of 5xTC150s that Vero had as extras and infected  
each with  $\approx$  0.7ml of ATCC P<sub>3</sub> Vero (moi of  $\approx$  0.001) - will leave  
over the weekend and harvest on Mon/Tues.

Meanwhile freeze down Vero clones ① and ② - Resuspended cell pellets  
in 90% FCS 10% DMSO after trypsinization and dilution in Growth medium.

Fed Vero clones 4, 5 and 6 with some fresh growth medium.

Passaged Vero clone 3 into 2x 6 well plates.

Set up TC150s on 32°C/37°C P<sub>3</sub> Vero infected  
Hep2/Hep2 morpho. 5 mins P<sub>3</sub> Vero inf.  
Vero clones 1/2 P<sub>3</sub> Vero infected

Stored Vero clones in Upright freezer.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kibbe*

Recorded by

*David K. Clarke*

000111

TITLE: \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

89

From Page No. \_\_\_\_\_

Informal interview with (Rong-guo Qiu) Ren Chiu

Very pleasant fellow, and well experienced in mol. biology.

Checked infected virus - They are already showing fairly good c.p.e. - It seems that slightly elevated temperature is better for RSV growth etc.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sever Koble*

Recorded by

*David K. Clarke*

000112

From Page No. \_\_\_\_\_

Preliminary conclusions from TCID<sub>50</sub>s

① Hep-2 cells bud virus out better than Vero

② The new Vero clone ① looks to be producing considerably more virus than the standard Vero cell.

Checked infected TC150s - They were blown away - there were no areas of cells remaining unaffected by virus c.p.e.  
 Harvested as per usual - total ≈ 90ml as (P<sub>3</sub> Vero ATCC 11-22-93) freeze down

Checked Vero clones - Clone ③ is ready to be infected and passaged

Infected V. clone ③ with 100 $\mu$ l of P<sub>3</sub> Vero (most recent from 5 x TC150s)Also passaged V. clone ③ into a 25cm<sup>2</sup> Flask.

adsorbed &amp; Shs at 37°C then added 2ml mem.

Set up Kinasing reaction on oligos 187, 188, 189  
 Used 25 $\mu$ l of each oligo

10m Spermidine

200 $\mu$ M ATP

X10 RIN buffer

1 $\mu$ l Enzyme in final vol of 50 $\mu$ l

1hr at 37°C. (actually 1 1/2 hrs!)

Am leaving: leader ① and leader ③ unkinased for ligation purposes.

After Kinase: RIN, phenol chloroform extracted and EtOH pptd.

Prepared X1TE buffer - autoclaved

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge W. Kibbe

Recorded by

David K. Clarke

0000113

TITLE \_\_\_\_\_

From Page No. ⑩

$T_m$  for annealing of ① and ⑤ (leader digox) is  $76^{\circ}\text{C}$  /

$T_m$  for annealing ④ to ①-⑤ is  $52^{\circ}\text{C}$

$T_m$  for annealing ② to ①-⑤-④ is  $76^{\circ}\text{C}$

$T_m$  for annealing ③ to 1-5-4-2 is  $50^{\circ}\text{C}$

Therefore will anneal ② to ④ separately and ① to ⑤ separately to accommodate higher  $T_m$ 's. Will then mix both together and anneal, finally adding ③ to complete the RSV end - Then Ppt and

↓  
equimolar amount (use less)  
 $\approx \frac{1}{2}$  amount

Ligate

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Khl*

Recorded by

*David K. Clarke*

0000114



From Page No. \_\_\_\_\_

Prepared 1% LMP Agarose gel ~~in preparation~~ to run out pUC 19 and gel purify.

Checked  $\beta$ -Vero infected Clone (3) - no sign of c.p.e. yet.

TCD<sub>50</sub> are still showing (1) a better yield with Vero clones (1) and (2) than with the usual Vero cells.  
(2) Much better yields from Hep-2 cells and budded out from these cells much better.

Spin down some of Kathy's Vaccinia DNA - will cut with Bam HI

Load 'F' protein in speed vac - Resuspended 'M' in 70% ethanol added NaCl and re-ppt'd at -10°C in order to get it into eppy tubes  
Spin down Sains - in microfuge - dried - resuspended in 50  $\mu$ l H<sub>2</sub>O

Set up Digest 25  $\mu$ l of 'M' DNA solution.  
5  $\mu$ l x10 buffer  
5  $\mu$ l BSA 100  $\mu$ g/ml  
12  $\mu$ l H<sub>2</sub>O  
3  $\mu$ l Enzyme.  
50  $\mu$ l - 2 hrs at 37°C

Run pUC 19 on a LMP agarose gel - Excise Band of DNA and run through  $\beta$ -agarose extraction procedure

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Sgt W. K. Khl*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

*David R. Clarke*

000115

TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

93

From Page No. \_\_\_\_\_

Vaccinia M. clone cut with Bam H.I.



Smear of vaccinia  
DNA + host DNA  
and some PCR

Will probably have to do a  
PCR rxn to amplify up  
the appropriate genes

However - will try to purify  
genome first

bRNA?

Completed purification of PUC19 DNA - stored at  $-10^{\circ}\text{C}$  as dried pellet - will  
resuspend at  $100\text{ng }\mu\text{l}^{-1}$  i.e.  $\approx 50\mu\text{l}$

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

*Steve W. Kibbe*

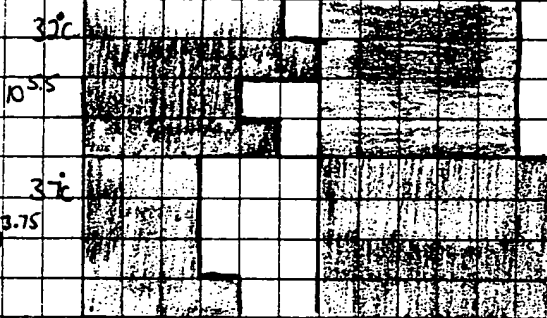
Recorded by \_\_\_\_\_

*David K. Clarke*

0000116

From Page No. \_\_\_\_\_

Did final Readings on TCID<sub>50</sub> Results are very interesting.  
All infections were with "Working stock" - ATCC P<sub>3</sub> Virus.



Hep-2 spin 10, using 5 min.

 $= 10^{5.5}$ 

Hep-2 total

 $= 10^{6.5}$ Will Reset incubator  
at 32.5 and doHep-2 experiment at  
32/37°

Vero Clone 1

 $10^{5.75}$  $10^{5.5}$ 

Clone 2



Ordered antileukemia (Bullock)  
cell scrapers  
\* Dispensator (Fisher)

Froze down Vero clone (3) with clones (1) and (2)

Fed clones (4) - (5) and (6)

Harvested virus from Clone (3) infected with RSV ATCC P<sub>3</sub> Virus  
After scraping cells (didn't get them all!) into  
supernatant - did a freeze-thaw and then  
spin down and aliquotted super C.P.E. could have been a little better (but the virus pretty good)  
prior to freezing

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne W. Kille

Recorded by

David K. Clever

TITLE: \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

95

From Page No. \_\_\_\_\_

Set up digestion of PUC 19 (gel purified) - started out with Kpn I  
digestion in 3 $\mu$ l final vol.

1.5 $\mu$ l ENZ.  
3 $\mu$ l x10 buff.  
3 $\mu$ l x10 BSA  
2.5 $\mu$ l H<sub>2</sub>O  
3 $\mu$ l

2hs at 37°C (12-45pm start)

Oligo annealing:-

Will do annealings in 0.1M NaCl, 10mM Tris, 1mM EDTA  
at appropriate T<sub>m</sub>. (Use small volumes - 30-60mins per annealing) 20-50 $\mu$ l at  
final stage

Will resuspend each oligo (Kpnased) in 10 $\mu$ l of H<sub>2</sub>O

Checked Vero clones - threw out ⑥ (appeared to be some contamination  
and the cells were not growing very well) Clone ⑤ appeared to be growing  
fairly well - so can probably split into 2 x 6 well dishes by tomorrow or today.

Heat inactivated Kpn I at 68° for 10-15mins - then set up Hind III digestion in  
3 $\mu$ l using 1 $\mu$ l enzyme.

Prepared a LMP 1% agarose gel and ran double digested PUC 19 DNA  
alongside a 1Kb DNA ladder - Excised  $\approx$  3Kb band and did agarose treatment.

Noted that there appeared to be a blurred or possibly doublet in the band  
of digested DNA - will run another gel tomorrow (regular 1%) and test a  
small sample of the eluted DNA. (About 1/10<sup>m</sup> should be enough).

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Sye W. Kluh*

Date

Invented by

Date

Recorded by

*David K. Clarke*

000118

From Page No. \_\_\_\_\_

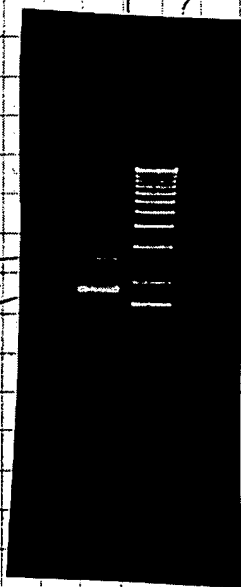
Prepared a 1% agarose gel for analysis of gel purified KpnI/HindIII at  
puc 19. Run  $\frac{1}{10}$  of the purified material.

Infected confluent Hep-2 cells with 0.25ml of 'old' P<sub>3</sub> Vero (ATCC).  
for 32°C/37°C experiment - absorbed both for 2-3hrs at 37°C then  
placed the '32°C flask' at 32°C

Meanwhile carried out TCID<sub>50</sub>s on Vero clone (3) Virus (V. clone (3) infected with  
P<sub>3</sub> Vero old)  
also did TCID<sub>50</sub> on 'New' Working Stock, P<sub>3</sub> Vero.

Split Vero clone (5) into 2 separate 6 well plates

KpnI/HindIII cut Puc 19  
ladder.



photographed digested puc 19 - it's  
not cutting efficiently!

will do digestion on mini prep DNA  
and etop ppt in between digestions

Spun down Kinased Oligos - did  
not see a visible 'pellet' in (189) well  
add more salt + glycogen to ensure  
ppt. Meanwhile dried the other two  
oligos and stored at -10°C.

Will add some more NaCl  
and my spinning down  
again tomorrow

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

*Steve W. Kuhl*

Recorded by \_\_\_\_\_

*David K. Clarke*

000119

TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

97

From Page No. 96

Will set up Kpn I digestion on remaining mini-prep DNA in 80  $\mu$ l final vol

80  $\mu$ l x10 buffer  
~~80  $\mu$ l~~  
8  $\mu$ l of  $10^6$  diluted BSA stock  
6  $\mu$ l  $H_2O$   
3  $\mu$ l Kpn I  
80  $\mu$ l ( $\frac{1}{2}$   $\mu$ l BSA stock - optional)  
Can control digestion in 20  $\mu$ l.

Phenol Chloroform extract - ppt at  $-10^\circ C$  ex and do Hind III digest tomorrow.  
Retained 4  $\mu$ l for gel analysis

~~Will~~ Will anneal 1 and 5 as follows - Mix 5  $\mu$ l of 190 (unknased) with 2.5  $\mu$ l of 187.  $76^\circ C$  for 10 mins - let cool slowly to  $60^\circ C$  - hold for 10 mins - let cool to  $50^\circ C$   $\rightarrow$  ice.

Respin 189 - looks as though some DNA came down - put back at  $-10^\circ C$  ex.  
Will spin again tomorrow

~~AK~~ NB Should try an infection of New Vero clones with a higher titer virus stock eg.

Witnessed &amp; Understood by me,

Date

Invented by

Date

To Page No. \_\_\_\_\_

*Sege W. Kuli*

Recorded by

*David K. Clarke*

0000120

From Page No. \_\_\_\_\_

Spin down Kpn I digested PUC 19 miniprep DNA - set up Hind III digestion in 80  $\mu$ l

8  $\mu$ l x10 buffer69  $\mu$ l H<sub>2</sub>O3  $\mu$ l Enzyme80  $\mu$ l

Set up Hind III control with  
Georges plasmid

Checked Infected Hep-2 cells; they are already showing some signs of c.p.e. will leave at least another 24 hrs. The level of c.p.e. at 32°C and 37°C appear to be similar, perhaps slightly greater at 32°C.

Need to Re-Kinase (189) - Do 10  $\mu$ g this time and add glycogen

Ran a 0.7% Agarose gel to check the digestion products

LHS: <sup>Kpn</sup> ~~had~~ / Kpn.Cm / Hind III.Cm / Kpn-PUC 19 / Kpn-Hind III-PUC 19  
<sub>Hind III cut</sub> <sub>Kpn I cut</sub> <sub>Hind III cut</sub> <sub>Kpn I cut</sub> <sub>Ladder</sub>



Kpn I / nor Hind III appeared  
to cut

Ordered 25  $\mu$ g of pUC 19  
from Pharmacia to solve the  
problem.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kibbe*

Recorded by

*David K. Clarke*

TITLE: \_\_\_\_\_

From Page No. 98

Set up an *Nco*I digestion on mini prep DNA which wouldn't cut with *Kpn*I and *Hind*III, to see if there is a 2<sup>nd</sup> contaminating plasmid in the mini-prep. i.e. there is no *Nco*I site in pUC19 and therefore it is the most likely contaminating plasmid.

Final check on infected Hep2 - cpe is progressing v well at both 32°C and 37°C.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by m-

*Sgt W. Kobb*

Date

Invented by

Date

Recorded by

*David K Clarke*

000122



From Page No. \_\_\_\_\_

Checked infected Hep-2 cells; CPE has become extensive, will harvest later this afternoon or tomorrow morning.

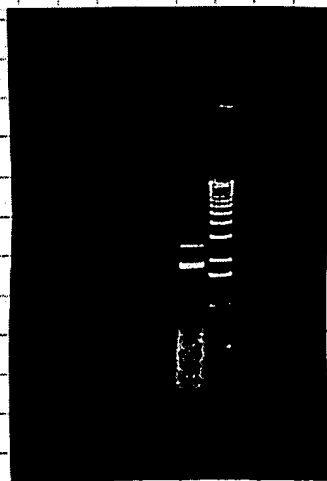
Meanwhile, set up Kinasig reaction on 189 for the 2<sup>nd</sup> time.

5  $\mu$ l x 10 buffer  
 3  $\mu$ l of 15mM spermidine (1mM final)  
 1  $\mu$ l of 10mM ATP (200  $\mu$ M final)  
 12  $\mu$ l Oligo (10  $\mu$ g total)  
 28  $\mu$ l H<sub>2</sub>O  
 1  $\mu$ l Enz  
 50  $\mu$ l 1 hr at 37°C

Phenol Chloroform extracted and stored at -10°C until Glycogen arrives.

Prepared and ran a 1% Agarose gel to analyse Nco I digestion products (from Mini prep DNA).

Checked Vero clone - will leave until Mon - then will re-feed.



Gel shows same DNA pattern for Nco I, as for Hind II and Kpn I.

Could be the DNA is modified or a potent inhibitor is present - can design an experiment to test this - add 60  $\mu$ l of Mini prep DNA to 8  $\mu$ l x 10 buffer 10  $\mu$ l of DNA (1  $\mu$ g  $\mu$ l<sup>-1</sup>) + 3  $\mu$ l enz.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Serge W. Kelle*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

*David K. Clatter*

TITLE: \_\_\_\_\_

From Page No. 100.

Harvested infected Hep-2 cells - Added Hepes/myso<sub>4</sub> after scraping cells into suspension; then went directly to freeze-thaw, followed by a spin - stored the super in 1ml aliquots at -80°C after freezing in ethanol/dry ice.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Sgt. W. Kille*

Date

Invented by

Date

Recorded by

*Warick K. Clarke*

000124

From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub>s - it appears that the New working stock is not  
V. good (perhaps the infection went too long and a lot of virus lost viability)

Also the titer from Vero clone ③ appears to be fairly good  $\approx 10^5$  (but not as  
good as clones ① and ②)

Set up TCID<sub>50</sub> for Hep-2 32°/37°C experiment - Plated out  
to  $10^{-7}$

Carried out purification of the Five oligos which I've cut out of a  
gel - Dried down

ppt<sup>d</sup> Spg of pUC-19 (from Pharmacia) for digestion with Kpn I. (Plat at 30°C  
for 1 hr)

(Spun down re-knused 189)

Ordered Dounce Homogenizer set; D. Spec. ordered gradient makers

Neil to copy Ward et al (N. caput/genomic RNA prep<sup>n</sup>) at UCSF.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Serge W. Kibbi*

Recorded by

*David R. Clarke*

TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

103

From Page No. \_\_\_\_\_

Spin down Spg of PUC 19 (Pharmacia) and set up Kpn I digestion -  
2 hrs at 37°C in 50 µl final vol.

5 µl x 10 buffer

5 µl x 10 BSA

38 µl H<sub>2</sub>O

2 µl enzyme

50 µl.

Then Phenol chloroform extracted and EtOH ppt.<sup>ed</sup>

Witnessed & Understood by me,

*Steve W. Kille*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

*David K. Clarke*

To Page No. \_\_\_\_\_

000126

From Page No. \_\_\_\_\_

Did final TCID<sub>50</sub> reading on Vero Clone ③ and New P<sub>3</sub> Vero ATCC working stock

10<sup>5.25</sup>

Vero Clone ③ infected with P<sub>3</sub> Vero ATCC  
(Not as good as Clones ① and ②.)

10<sup>5.5</sup>

'New' P<sub>3</sub> Vero working stock ATCC - comparable to  
'Old' P<sub>3</sub> Vero working stock - will use this for a  
N capsid prep/virus prep next MON on the following  
MON.

Set up Hind III digestion on Kpn I digested PUC19 (kept 1 $\mu$ l of Kpn I cut material for gel analysis)

5 $\mu$ l x 10 buffer  
43 $\mu$ l H<sub>2</sub>O  
2 $\mu$ l enz  
50 $\mu$ l

2 hrs at 37°C off at 12:30pm

Prepared a 1% agarose gel for analysis of digestion products

Infected 1x25cm flask of Vero and 1x25cm<sup>2</sup> flask of Hep-2 cell, each with 0.5ml of 9320 B virus adsorbed 3hrs (2-15pm feed)

Resuspended Tailer-oligos in H<sub>2</sub>O and measured Spk in lml to get an estimate of quantity (Watch 2016 carefully!)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Sege W. Kbl*

Date

Invented by

Date

Recorded by

*David K. Clarke*

0000127

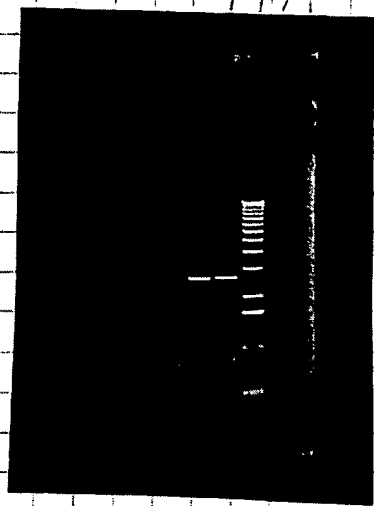
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Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

105

From Page No. 104.



Phenol chromform extracted and  
EtOH pptd.

EtOH pptd. header oligo.  
(kinased) 189 w/thout glycogen  
to see if it will come down.

Witnessed & Understood by me,

Date

Invented by

Date

To Page No. \_\_\_\_\_

*Sign W. K. Kble*

Recorded by

*Harold K. Clarke*

000128

From Page No. \_\_\_\_\_

Prepared lower mp agarose gel for purification of KpnI/HindIII cut PUC19 Vector.

Loaded gel and Ran at 50mA

Set up Kinasing of trailer oligos (except 201 and 197) - left unkinased at ends of trailer.

50µl final vol.

10µl copy of oligo in 6-35µl H<sub>2</sub>O

5µl x10

3µl 15mM spermidine

4µl 10mM ATP

H<sub>2</sub>O to 50µl

1µl Enzyme

11:15am - 12:15pm → ~~Ø~~ chloroform

Spun down Kinased leader (ppted yesterday)

Evap pptd (niglycerol)

Checked Vero clone (5) - needs to be fed - slow growing - may not be very

productive for virus

Checked c.p.e. on 'B' virus infected Vero and Hep-2s - as yet there is no obvious c.p.e. on either cell monolayer - tomorrow will be a more 'acid' test.

Continued purification of PUC19 on a gel - phenol chloroform extracted prior to evaporational ppt. step - spun down, and resuspended in a total vol. of 20µl H<sub>2</sub>O and froze at -10°C.

Interviewed Ben Chiu

See Reference binder #22A

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Steve W. Kelle

Recorded by

David K. Clarke

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

Checked c.p.e. on Vero/Hep-2 cells with 'B' virus.  
There is no obvious c.p.e. yet - may change medium tomorrow to see if that will stimulate things.

Did find read on TCID<sub>50</sub>s

-1 -2 -3 -4 -5 -6 -7 -8



26 = 10<sup>7</sup>

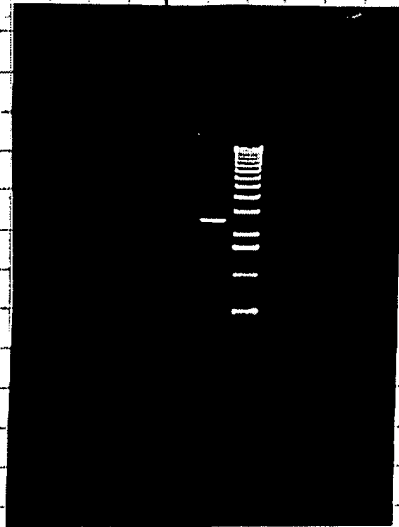
Hep-2 - infected with P<sub>3</sub>V 32°C. Highest titre obtained so far with P<sub>3</sub>V.

23 = 10<sup>6.25</sup>

Hep-2 infected with P<sub>3</sub>V 37°C

Prepared a 1% Agarose gel, and run out  $\approx$  1  $\mu$ l of gel purified Puc 19 (from 20  $\mu$ l) to check amount and efficiency of purification etc.

Prepared Leader annealing mixtures



1% Agarose gel of Gel purified

Puc 19

50 ng  $\approx$  1/20

$\approx$  1-2 ng of DNA

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Sgt. W. Kibbi*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

*David K. Clarke*

0000130



From Page No. \_\_\_\_\_

Carried out annealing of leader Oligos - forgot to use Kinased fragments appropriately, but will run on a 1% agarose gel to check how well they have annealed.

Prepared 1% gel - run - photographed.

Meanwhile infected 2x Hep-2 with 0.25ml of P<sub>3</sub>VPH-2 and 0.25ml of "New Working stock" P<sub>3</sub>Vero adsorbed 3hrs at 6-15pm

Oligo annealing (Leader)

Added 1.5µl of 190 ① and 1.2µl of 187 ⑤ in 10µl (using XIONTE)  
 Equimolar ratio.  
 unkinased                      kinased. (500µg µl<sup>-1</sup>)

Hold at 76°C 10mins  
 cool to 70°C 5mins  
 Hold at 70°C 10mins  
 cool to 65°C 5mins  
 Hold at 65°C 10mins  
 cool to 60°C 5mins  
 Hold at 60°C 10mins  
 cool to 55°C 5mins  
 Hold at 55°C 10mins  
 cool to 50°C 5mins  
 Hold at 50°C 10mins

Oligos ② and ④ can be  
 188                      189  
 Kinased                      Kinased

annealed at the same temperatures

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Suzanne W. Kuhl

Date

Invented by

Date

Recorded by

Daniel K. Charlie

0000131

TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

109

From Page No. 108

To anneal (V5) to (2/4) + Oligo (3) 186

unkinased

Hold at 55°C 10 mins

Cool to 50°C 5 mins

Hold at 50°C 10 mins

Cool to 45°C 5 mins

Hold at 45°C 10 mins

Cool to 40°C 5 mins

Hold at 40°C 10 mins

Annealing gel looks  
pretty good - the Oligo band is at  
the correct position.

Witnessed &amp; Understood by me,

*Sgt. W. K. M.*

Date

Invented by

Date

Recorded by

*Dwight R. Clatter*

To Page No. \_\_\_\_\_

000152

From Page No. \_\_\_\_\_

Checked cells infected with B' virus - still no major CPE detectable  
 Changed the medium and incubated at 37°C for a further 24-48 hrs.

①③ Ordered Potassium Tartrate.

Prepared 10ml of boiled glycogen (1mg/ml)

Took 1ml and phenol/chloroform extracted for RNA work

Prepared a 1% Agarose gel and ran out annealed leader Oligos for  
 Electrophoretic purification. EtBr stained - cut out annealed band and  
 electrophoreted - Phenol chloroform extracted and EtOH ppt'd.  
 Will check efficiency again tomorrow.

into TE  
 buffer

With some  
 Glycogen

(Salt conc may  
 have been too low)

Meanwhile prepared NA45 paper according to protocol;  
 also prepared N.E.T. and High salt NET

Checked infected HEP-2 cells - many cells appear to be dying - will leave  
 another 24hrs before harvesting and doing the TCID<sub>50</sub>s. (More look dead with higher  
 later input virus)

Autoclaved NET and high salt NET

Trailer oligos

⑥ = 206 Kinased

① = 201 Unkinased

③ = 205 Kinased

⑨ = 198 Kinased

⑧ = 204 Kinased

⑤ = 197 Unkinased

⑦ = 203 Kinased

④ = 213 Kinased

② = 202 Kinased

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kille*

Recorded by

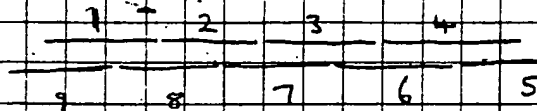
*David R. Clarke*

000133

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Plan for Trailer annealing

Resuspend Kinased oligos  
at 500 µg ml<sup>-1</sup>\* 1:7 annealing  $T_m = 56^\circ\text{C}$ 1:8 annealing  $T_m = 52^\circ\text{C}$ 2:8 annealing  $T_m = 64^\circ\text{C}$ 2:7 annealing  $T_m = 62^\circ\text{C}$ 3:7 annealing  $T_m = 62^\circ\text{C}$ 3:6 annealing  $T_m = 62^\circ\text{C}$ 4:6 annealing  $T_m = 60^\circ\text{C}$ \* 4:5 annealing  $T_m = 56^\circ\text{C}$ 

Anneal 2, 3 and 7 together in 1<sup>st</sup> run  
Then add 8, and 6 for 2<sup>nd</sup> run. Then add  
4 for 3<sup>rd</sup> run, then 2 for 4<sup>th</sup> run and then  
5 and 9 for 5<sup>th</sup> and final run.

Use µg of each oligo

1<sup>st</sup> run.Hold at  $62^\circ\text{C}$  10 minsCool to  $57^\circ\text{C}$  5 minsHold at  $57^\circ\text{C}$  10 minsCool to  $52^\circ\text{C}$  5 minsHold at  $52^\circ\text{C}$  10 minsCool to  $47^\circ\text{C}$  5 minsHold at  $47^\circ\text{C}$  10 minsCool to  $42^\circ\text{C}$  5 minsHold at  $42^\circ\text{C}$  10 mins

\* add last in annealing - both together?

Do 10 µg total.

2<sup>nd</sup> RunRepeat of 1<sup>st</sup> Run3<sup>rd</sup> RunHold at  $60^\circ\text{C}$  10 minsCool to  $55^\circ\text{C}$  5 minsHold at  $55^\circ\text{C}$  10 minsCool to  $50^\circ\text{C}$  5 minsHold at  $50^\circ\text{C}$  10 minsCool to  $45^\circ\text{C}$  5 minsHold at  $45^\circ\text{C}$  10 minsCool to  $40^\circ\text{C}$  5 minsHold at  $40^\circ\text{C}$  10 mins4<sup>th</sup> Run.  $52^\circ\text{C}$  10 minsCool to  $47^\circ\text{C}$  5 mins47 $^\circ\text{C}$  10 minsCool to  $42^\circ\text{C}$  5 mins42 $^\circ\text{C}$  10 minsCool to  $37^\circ\text{C}$  5 mins37 $^\circ\text{C}$  10 minsCool to  $32^\circ\text{C}$  5 mins32 $^\circ\text{C}$  10 mins5<sup>th</sup> RunRepeat of 4<sup>th</sup> Run.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Sgt. W. K. Kuhl

Date

Invented by

recorded by

David K. Clarke

Date

0000134

From Page No. \_\_\_\_\_

Checked 'R' infected cells - still no sign of CPE - will leave until tomorrow then harvest and make TCID<sub>50</sub> measurement

Set up leader annealing one more - this time using Kinased oligos

Mixed 1.2 (1.5  $\mu$ l) of unkinased 190 with 1.2  $\mu$ l (2.4  $\mu$ l) Kinased 187 added 1  $\mu$ l NTE and 5.4  $\mu$ l H<sub>2</sub>O  $\rightarrow$  10  $\mu$ l. Then ran annealing program #60 as before.

Meanwhile prepared 1% agarose gel for analysis of the annealing products + yesterday's electrophoresis procedure.

Did likewise for Oligos (2) 188 Kinased and (4) 189 Kinased.

Then mixed both annealed samples and added (3) 186 unkinased and went through program "61" 55°C annealing  $\rightarrow$ .

Spin down  $\frac{1}{2}$  of electrophoresed annealed leader - resuspended in 20  $\mu$ l H<sub>2</sub>O, and ran  $\frac{1}{2}$  of that on a 1% agarose gel along with  $\frac{1}{2}$   $\mu$ l from annealed mixture (which had been kinased).

Also harvested Hep-2s infected with V<sub>3</sub>H<sub>2</sub> and New V<sub>3</sub> <sup>grown</sup> at 32°C

$\downarrow$   
V<sub>3</sub>H<sub>2</sub>

$\downarrow$   
V<sub>3</sub>H<sub>1</sub>

Freeze down - will do TCID<sub>50</sub> on Wed.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Sege W. Kblh*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

*David K. Clarke*

000135

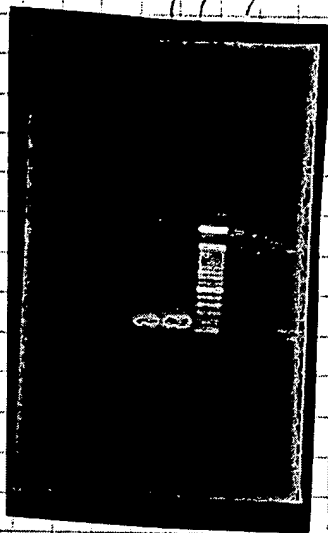
TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

113

From Page No. \_\_\_\_\_



Never 3 binder  
Older  
noob platter

Older annealed algo has  
held up well

looks encouraging

Witnessed & Understood by me,

Date

Invented by

Date

To Page No. \_\_\_\_\_

*Sgt. W. K. White*

Recorded by

*David R. Clarke*

000136

From Page No. \_\_\_\_\_

Checked c.p.e. on B 9320 infected cells - may be some on Vero -  
harvested both Hep-2 and Vero (5 days post infection) - carried out TCID<sub>50</sub>  
(out to  $10^{-3}$  dil<sup>n</sup>) for both harvests.

Also did TCID<sub>50</sub> on (New) P<sub>3</sub>V P<sub>1</sub>H and P<sub>3</sub>V P<sub>2</sub>H

Checked Vero clone ⑤ - growing fairly well - will sub culture into a 25cm<sup>2</sup>  
flask on Mon. and feed duplicate prior to infection later this week.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Steve W. Kuhl*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

*David K. Clarke*

000137

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Infected 10 x TC 150s (70-80% confluent) each with  $\pm$  1 ml of  
Old P<sub>3</sub> Vero and some New P<sub>3</sub> Vero 10am - adsorbed 3 hrs  $\rightarrow$  1pm then  
Fed with 20ml MEM.

Spun down Kinased Oligos  $\rightarrow$  visible pellet - resuspended in 20  $\mu$ l H<sub>2</sub>O at  
 $\approx$  500ng  $\mu$ l<sup>-1</sup>

Set up 1<sup>st</sup> step in annealing trailer region

Mixed 2.3  $\mu$ l ( $\pm$  1.2  $\mu$ l) each of Oligos 205, 203, 202, in x1 NTC  
with a final vol of 10  $\mu$ l - (see page 114)

Step 2 was identical with the addition of Oligos (6) + (8)

Step 3 Oligo 4 was added

Step 4 Oligo 1<sup>\*</sup> was added or unkinased

Step 5 Oligos 5 and 9

Stored at -10°C prior to ligation/gel purification

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Steve W. Koble*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

*David K. Clarke*

0000138



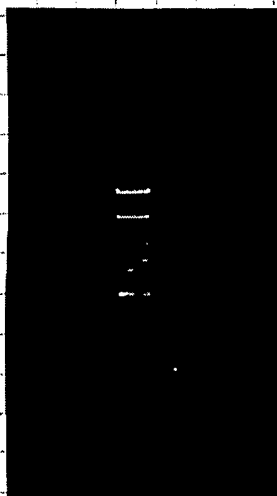
From Page No. \_\_\_\_\_

Prepared 1% agarose gel for analysis of trailer annealed products

Run - stained - photographed

Gel shows  $\approx$  200bp fragment in the approx correct position for the complete leader trailer region.

Will set up a ligation with 30  $\mu$ g of CAT gene and 10 fold molar excess of leader and trailer. Will need 1.2  $\mu$ g of leader (K<sup>+</sup> K<sup>-</sup>) and 2.4  $\mu$ g of trailer.



200ng.

Checked infected Hep-2 cells - not too much c.p.e. visible yet - some syncytia are visible. Will check again this evening.

Checked gradient maker - can probably prepare 4ml 15-55% gradients if use 2.5ml in each chamber.

Ordered SG5 and SG15 (5ml and 15ml) gradient makers.

Will use 4  $\mu$ l from each leader prep K<sup>+</sup> K<sup>-</sup> and ppt with 1/2 of trailer prep and 1/2 of CAT gene prep and then set up ligation.

Mixed  
10  $\mu$ l of annealed trailer  
1  $\mu$ l of annealed leader  
10  $\mu$ l of CAT gene  
and ppt<sup>21</sup> all together at -80°C - then set up ligation in 40  $\mu$ l final vol.

4  $\mu$ l x 10 buffer  
3  $\mu$ l H<sub>2</sub>O  
2  $\mu$ l ligase

40  $\mu$ l

Store ligation at -10°C up to prior to gel analysis

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sege W. Kibbe*

Recorded by

*David K. Clarke*

000139

From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub>s - it appears that higher input does give a higher yield of virus but overall yields appear to be a little lower than expected perhaps due to the fact that the Heaps were a little overgrown when infected.

Will make a final read on Fri.

② M / <sup>correct</sup> ~~higher~~

Should set up another ligation with increased amounts of CAT + leader (keep trailer the same or a little less)



Gel shows products of ligations containing CAT gene, Trailer and leader from RSV

Clearly CAT gene was limiting but some of the correct sized material is present

PUC19 is ~3 times the size of the CAT/RSV construct. Will also use in 2x excess of PUC to favor capture of the appropriate DNA

Would estimate 10-20 ng /  $\mu$ l for correct species of DNA  
in 4  $\mu$ l = 100-200 ng

Will use 25-50 ng of CAT/RSV and ~200 ng of Vector PUC.  
-300 ng  
2/3 of total prep

Also harvested 10x TCID<sub>50</sub>s after 4 hrs p.i.  
(Numerous syncytia were visible)

Went through N. capsid preparation scheme

Spin 15 mins at 3000 x g for MEM super. - kept pellets and spin this super at 27,000 rpm in SW 28 4°C for 1.5 hrs to pellet 'free' virus - pellets looked good - (small)  
Removed cell membranes with 'Versene' and pelleted on top of 1st super pellets - then hook up 2 pellets in lysis buffer (add small vol. 15%) - Ice 20 mins - Dance 10 strokes; spin out nuclei etc 1.75K 2 mins - Pelleted N. capsid from super 10K JA20 4°C - lysed (broken) in lysis buffer - repelleted, washed with T.E - repelleted - resuspended in 15% K Turbinate (w/w in T.E) - Very difficult to resuspend (use larger Vol. next time + a little D.O.C? sis?)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sege W. Kibbe*

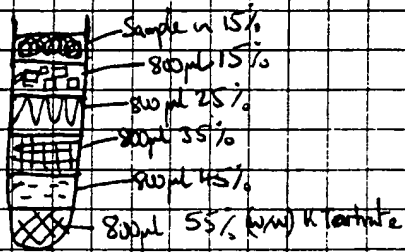
Recorded by

*Wendel K. Clarke*

0000140

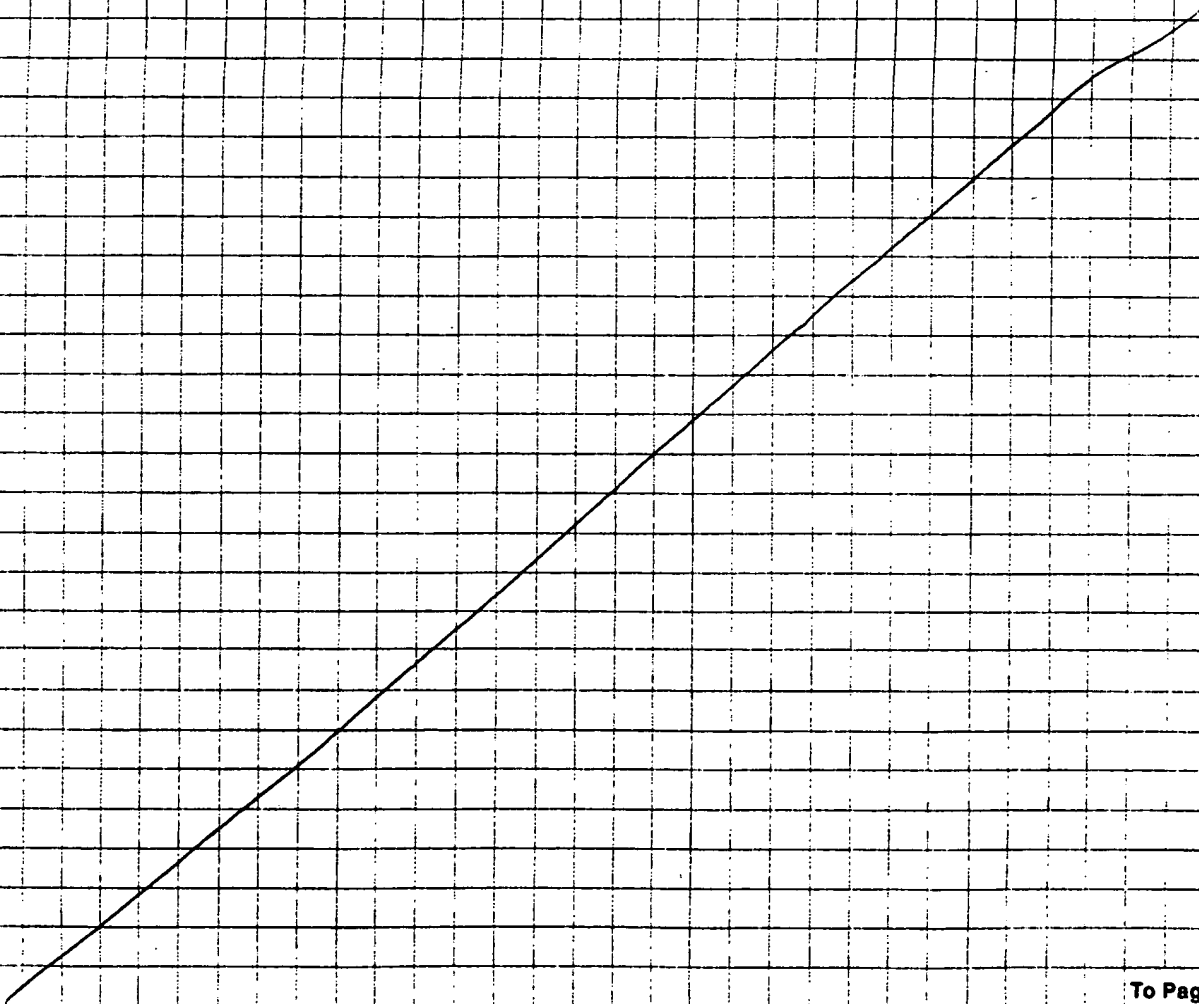
From Page No. 117.

Prepared a total of 6  $K^+$  Tartrate step-gradients in SW 50.1 tubes



Spun at 29K for 16 hrs at 5°C  
in SW 50.1

Meanwhile resuspended Virus pellet (each in 100 µl of NTE x1) on ice (at 4°C) O.M.



To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Steve W. Kline*

Date

Invented by

Date

Recorded by

*Dwight K. Wether*

000141

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Took off N capid spin. There was a band of white somewhat flocculent material a little over  $\frac{1}{2}$  way down the gradient. Harvested all 6 gradients - diluted x4 in T.E. and pelleted white material in the eppendorf (10 min spin). Poured off super - rinsed with T.E. and then resuspended pellets in 200  $\mu$ l T.E. each - added SDS to 0.2% and X10 Proteinase K left 1.5 hr at 37°C (all floccs disappeared).

Then phenol chloroform extracted (also ended up doing x2). Rinsed x2 with chloroform and ppt'd with 2 vols of EtOH.

Meanwhile resuspended Virion pellets - did SDS/proteinase K - phenol chloroform extraction and etOH ppt'd.

Set up ligations

- ① 10  $\mu$ l of  $K^+$  leader ligation containing some of correct sized plasmid construct.  
 5  $\mu$ l of PUC  $\approx$  25 ng  
 2  $\mu$ l x10 buffer  
 2  $\mu$ l H<sub>2</sub>O  
 1  $\mu$ l T<sub>4</sub> ligase  
 20  $\mu$ l.

- ② 15  $\mu$ l cat. gene  
 8  $\mu$ l Annealed  $K^+$  trailer  
 8  $\mu$ l "  $K^+$  leader.

Put both ligations at 37°C for  $\approx$  45 mins - then left at R temp 90.

ppt'd together - 70% EtOH rinse - dried  
 then 4  $\mu$ l x10 buffer  
 3  $\mu$ l H<sub>2</sub>O  
 2  $\mu$ l ligase.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Suzanne W. Kibbi

Date

Invented by

Date

Recorded by

David &amp; Clarke

000142

From Page No. \_\_\_\_\_

Prepared 1% agarose gel for analysis of ligation products - will look at type of ligation mixture.

Prepared 200ml of 1-Agar Amp<sup>r</sup> for Transformation

-1 -2 -3 -4 -5 -6 -7 -8

10<sup>7</sup>V<sub>3</sub>H<sub>2</sub> 32°C

Cells were overgrown - which probably had a significant effect - will repeat some with more normal Hep-cell monolayers.

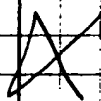
10<sup>6</sup>New V<sub>3</sub>H<sub>1</sub> 32°C

Clearly a 10X difference in virus stocks though!

-1 -2 -3 -4

9320 p.v.  
37°C

B-virus is growing (puck) on Veroes but not at all on Hep's!



9320 p. Hep's - didn't grow!

Set up transformation using 2 50µl competent cells/tube

EFF. control (1µl of control DNA)

CAT DNA con. (1µl of purified CAT)

Cut pUC con (1µl of purified stuff)

Ligation Mixture 5µl of 20µl

Inc 1.5 hrs

from 42°C heat shock

0.9ml SOC. 2 hrs at 37°C

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Koble*

Recorded by

*Harold - K. Clarke*

000143

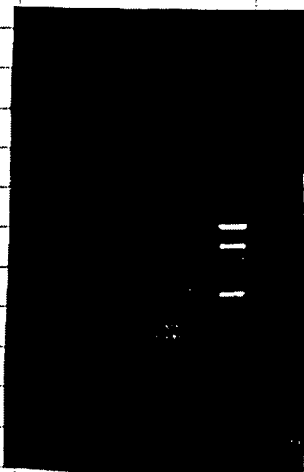
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Project No. \_\_\_\_\_

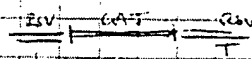
Book No. \_\_\_\_\_

121

From Page No. \_\_\_\_\_



New ligation conditions have  
pushed more CAT gene into

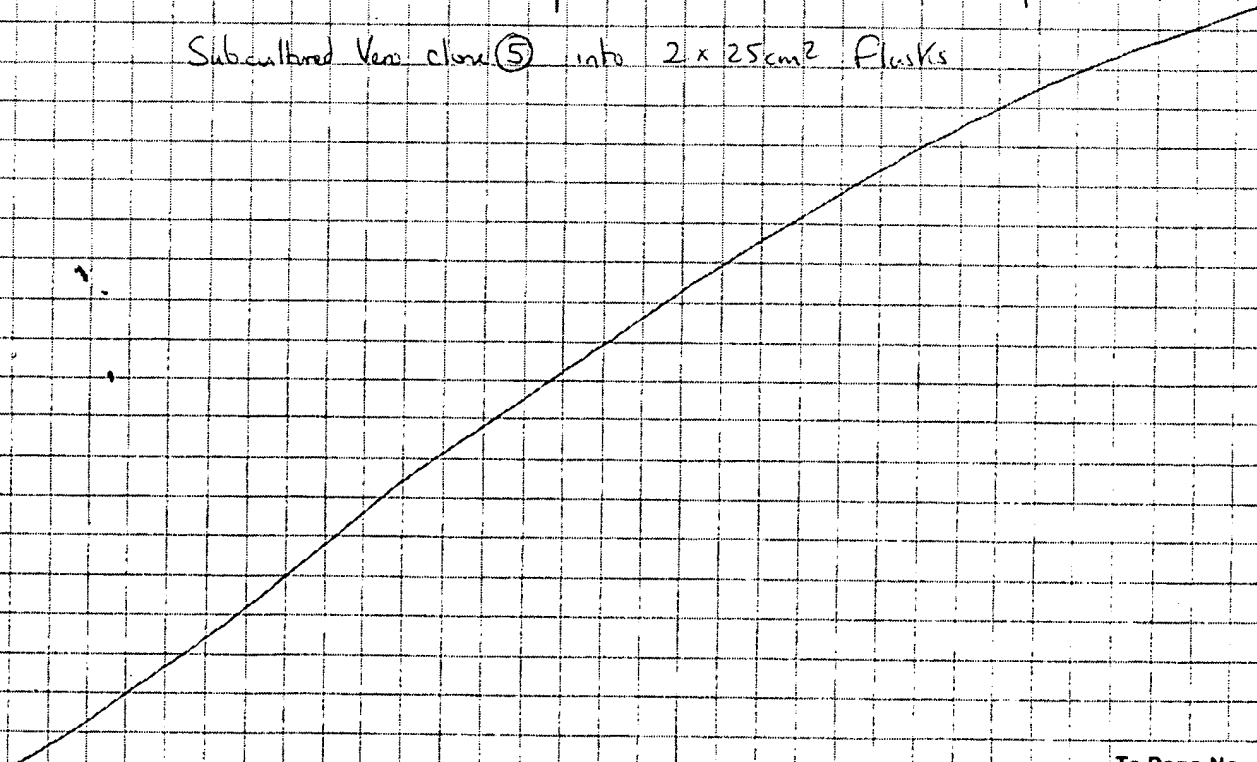


perhaps 30-40% in  $\frac{1}{10}$  of  
ligation mixture = 300-400 total

plated transformants on L-Agar Amp<sup>r</sup> ( $10^6 \text{ mg ml}^{-1}$ ) containing 80  $\mu\text{L}$  of X-gal stock  
and 8  $\mu\text{L}$  of IPTG.

Put 200  $\mu\text{L}$  of transformed cells on each plate

Subcultured Vero clone ⑤ into  $2 \times 25 \text{ cm}^2$  Flasks



To Page No. \_\_\_\_\_

Witnessed & Understood by me, \_\_\_\_\_

*Steve W. Kibbe*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Recorded by \_\_\_\_\_

*David K. Clarke*

Date \_\_\_\_\_

000144

From Page No. \_\_\_\_\_

Checked transformed cells - lots of white colonies even on the PUC transformed control plate - seems like Blues are secreting  $\beta$ -lactamase allowing satellites to spring up.

CAT DNA alone - Zero colonies - No blues : No whites!  
 Eff. con - quite a few blues - many whites  
 Cut PUC - quite a few blues - many whites  
 Ligate mixture - Very many Blues and Whites

Will repeat on Monday - using 50  $\mu$ g/ml Amp plates and a dilution series of ligation mixture (undil. 2  $\mu$ l,  $10^{-1}$  20  $\mu$ l,  $10^{-2}$  20  $\mu$ l)

Will transform with 4  $\mu$ l of ligation mixture.

The <sup>large</sup> increase in number of blues in the ligation mixture transformation over the cut PUC (a similar total amount of PUC in each case) indicates that the ligation did work and regenerated plasmid where only single enzyme cuts had occurred. (either KpnI or HindIII)

It remains to be seen if some double cut plasmid picked up the RSV/CAT DNA

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,



Date

Invented by

Date

Recorded by



0000145

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Prepared 200ml L-Agar - poured plates x6 containing 150µg ml<sup>-1</sup> Amp

In the meantime infected 10 x TC 225 - each with 1.5ml of 'New' working stock (p3V A1cc) Adsorbed ≈ 3hrs.

Checked Vero clones - not yet confluent - will infect later this week - also will freeze down one flask.

Set up transformation 50µl competent cells 100µl for ligation mixture  
1µl of either Control 1µl ligation mix  
pUC or Cut pUC.

left on Ice for ≈ 1hr. Lysed 42°C heat shock - 1hr 37°C with 0.9ml SOC.

Infected a 25cm<sup>2</sup> Vero monolayer with ≈ 1ml of P: Vero 'B' 9320 adsorbed ≈ 3hrs then added ≈ 3ml of MEM.

Prepared IPTG & 1a Mannitol in sterile H<sub>2</sub>O - filter sterilized - aliquotted and froze at -20°C.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sege W. Kuhl*

Recorded by

*David K. Clarke*

000146



From Page No. \_\_\_\_\_

Checked transformations - No breakthrough colonies this time - however there are as many white colonies on the Unligated PUC (cont) as on the "no ligat" mixture.

Ligation regenerated many blue colonies - indicating that a large proportion of plasmid was cut only once or that many doublets were formed from Head-to-tail ligation of PUC.

Will streak out whites on an amp<sup>r</sup> Mac<sup>r</sup> + Chloramphenicol<sup>r</sup> plate. Will also phosphorylate PUC and Kinase ligation products - Would not be optimistic about any of the whites since there was an equivalent amount of PUC (and equiv. number of whites) in the Con. and Test transformations.

Prepared Cm<sup>r</sup> Agar - poured - dried - plated out Bays.

Checked infected Veroes + itops - No signs of major c.p.e. yet - will check again at 5-30pm.

Phenol Chloroform extracted ligation mixture and then set up Kinase reaction - Ppt<sup>d</sup> PUC DNA with a little glycogen and set up phosphorylation reaction.

Ham. SAP run on.

After both reactions, phenol chloroform extracted and ppt<sup>d</sup>.

(Set up Q/N ligation reaction utilizing  $\frac{1}{2}$  of PUC 19.2)

Will gel purify ligation products before setting up ligation and run  $\frac{1}{4}$  of gel purified material to check % recovery.

Streaked out Bacterial transformants on Cm<sup>r</sup> and AMP<sup>r</sup> plates and grew Q/N at 37°C.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Suzanne W. Koble*

Recorded by

*David R. Clarke*

0000147

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Checked bacterial transformants - 3 showed a little growth on  $\text{Cm}^+$  plates - may not be significant - respread and incubated at  $37^\circ\text{C}$  O/N once more. Kept the Amp $^+$  plates at room temp. to allow Blue colour to finally develop.

Went ahead with virus harvest - Clarified supernatant - 20 mins at 3K then proceeded with high-speed spin. (C.P.E. was well advanced on Hepc - 48h p.i.) - had to discard some of the super as did only one run on the SW 28.

Proceeded to take off cells with Versene - spin down at 3K for 15 mins - decanted super and froze cell pellets until tomorrow.

Put on Virus spin on refurbished centrifuge - 27K rpm  $4^\circ\text{C}$  1.5 hrs.

Meanwhile spin down CAT/leader/Trailer DNA and electroeluted from a  $1\frac{1}{2}$  Agarose gel.

Checked clones that were showing signs of growth on chloramphenicol plates - they are showing signs of continued growth - will set up 4 mini preps O/N - 3 potential trees and 1 neg.

Centrifuge is working fine - took off spin - decanted super, and drained thoroughly - then resuspended on in 100  $\mu\text{l}$  of NTE.

Used NA+S paper - eluted into 100  $\mu\text{l}$  of High Salt NET. Diluted to 0.5M NaCl - added 2  $\mu\text{l}$  of glycogen (20%) and EtOH ppt $^d$ .

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sgt. W. K. K. K.

Recorded by

David K. Clarke.

000148

From Page No. \_\_\_\_\_

Removed  $Cm^R$  bacteria from incubator - growth looked encouraging.

Went through mini-preps to check for CAT/rev clones

Meanwhile prepared  $40\%$  -  $30\%$  -  $25\%$  -  $5\%$  sucrose gradient for

isolation of nucleocapsid & la Udem and Cook. Introduced the Dancie homogenizer procedure from the Mike Mink protocol, but otherwise was as published in Udem and Cook.

Set up mini-prep digests on  $\frac{1}{2}$  of total DNA in each case

29  $\mu$ l H<sub>2</sub>O

5  $\mu$ l x 10 BSA

5  $\mu$ l x 10 RXN buffer

1  $\mu$ l DNA

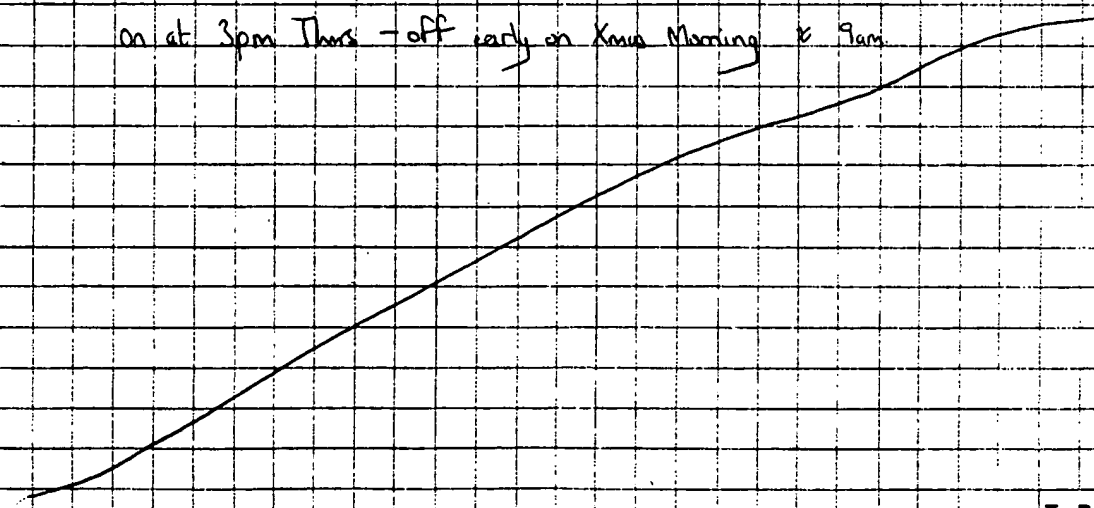
1  $\mu$ l Enzyme

50  $\mu$ l 37°C for 1.5 hrs.

- ppted at -20°C ON after digestion prior to Hmd III digestion.

Set up Isopycnic gradients (x2) for Nucleocapsid in SW50.1 38K for 36 hrs

on at 3pm Thurs - off early on Xmas Morning @ 9am



To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*David W. K. Clark*

Recorded by

*David K. Clarke*

000149

# Identification of Potential CAT/RSV constructs.

TITLE Preparation of genomic RNA from Virions

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

127

From Page No. \_\_\_\_\_

Set up Hind III digestions on mini preps - previously cut with Kpn I - to check for CAT/RSV insert - prepared a 1% agarose gel for analysis of digestion products. Also spun down NAKS-paper purified CAT/RSV DNA from ligations done earlier in the week - will analysed 1/4 of total on the gel.

Meanwhile did proteinase K, SDS digestion of resuspended Virions; then proceeded with Phenol chloroform extraction, and ethanol ppt<sup>n</sup>.

Froze down Vero clone 5 in 90% FCS (heat inactivated) and 10% DMSO.

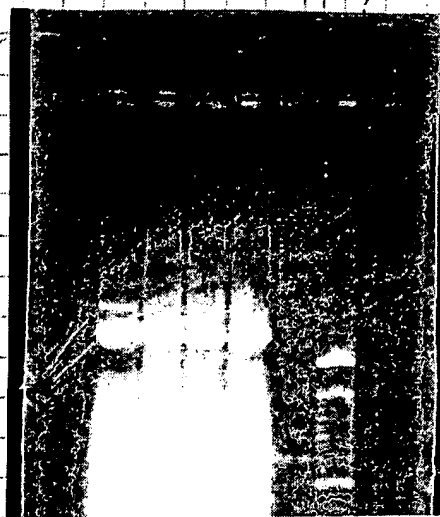
Changed medium on 'B' 9320 infected Vero, in an attempt to stimulate virus reproduction (3 days p.i.)

Split Hep 2s 1 to 4 (They use 10% FCS)

Split Vero 1 to 10

Mini preps

Eluted cat/RSV ladder



Mini preps do not show desired band.

Will proceed with ligation using 5 $\mu$ l of RSV/CAT ( $\approx$  25ng) and 7.5 $\mu$ l of PUC vector (use  $\approx$  1/2 of phosphorylated PUC). Run religated PUC con.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sgt W. K. K. K.*

Recorded by

*David K. Clarke*

0000150

From Page No. \_\_\_\_\_

Took off Cell spin (Nucleocapsid) - No N capsid band was visible, however a distinct RNA pellet was visible at the bottom of the tubes - these pellets were dissolved in 200  $\mu$ l  $H_2O$  - phenol chloroform extracted and EtBr pptd. The RNA may be Ribosomal RNA, but there could also be some N capsid RNA which lost the 'N' protein due to the high salt (GCL) and SES. Will try RT-PCR on a representative sample from the various preps.

Checked 'B' 9520 infected Vero's - They are showing good signs of c.p.e. (after the medium change at 3 days p.i.)

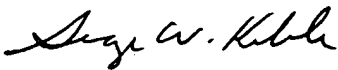
To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

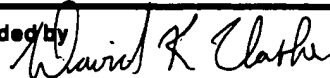
Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_



recorded by \_\_\_\_\_



CAT/RSV DNA Ligation to pUC 19.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

129

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Checked R9320 infected Vero's - C.P.E. now well advanced - decided to harvest; however all dry-ice and liquid N<sub>2</sub> was used up so decided to continue incubation at -32°C until tomorrow, when supplies will hopefully be restored.

In the meantime set up ligation of NA45 paper-purified <sup>Kinased</sup> CAT/RSV and SAP<sup>+</sup> pUC 19.

25 µl x 5 µl of CAT/RSV

75 µl 1/4" PUC prep

20 µl final vol

1 µl of T<sub>4</sub> DNA ligase 14°C ON

Also ran a 'PUC 19 (SAP<sup>+</sup>)' ligation control with no insert DNA.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sgt. W. Kelle*

Recorded by

*David K. Clarke*

000152

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Transformation with RSV/CAT/PUC19.

Control RT-PCR Using Neils' RNA + Primers.

From Page No. \_\_\_\_\_

Checked B9320 infected Vero - c.p.c. now very extensive - will harvest today as soon as the dry ice arrives.

Vero and HEPs need to be passaged today - also need to infect Vero clone (5) with 'working stock'.

Set up cDNA synthesis using Neils' RNA and primers (3 + 5)

(0.5µg) Spl RNA + Spl H<sub>2</sub>O, 92°C for 2-3 min to denature -

Snap cooled in ice bath to prevent reannealing

1µl Methylmercury 14mM → Same R-Temp.

3µl 2-mercaptoethanol 1.85M → Same R-Temp.

Used 1µl of Neils' Primer N° (3) for reverse transcription

Used 1µl of Reverse Transcriptase 1.5hrs at 42°C

Inactivated RTase at 94°C for 5 min.

Then set up PCR RXN - added 2µl each of Neils' primers (3/5) and 1µl Taq pol. + Mineral oil.

30 cycles  
 94°C 1 min 2 min  
 40°C 2 min 2 min  
 72°C 3 min

Meanwhile did transformation with PUC19 ligated to NA45 purified CAT/RSV (Kinase<sup>+</sup>)  
 added 5µl of ligation mixture to Invitrogen competent cells  
 - also did control with ligated PUC19 alone  
 - also tried transformation with some of Neils' competent DH5αs

Infected Vero clone (5) with 0.25ml of 'Working Stock' - started 2 Shrs at 37°C

Passed Vero and HEPs - will passage again on Wed or Thur and  
 Harvested P.V. B9320 - stored at -80°C

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

David W. Koble

Date

Invented by

Date

Recorded by

David K. Clarke

000153

TITLE \_\_\_\_\_

From Page # - \_\_\_\_\_

Checked Bacterial transformations  
from yesterday.

Results look encouraging. There are  
virtually no colonies on the control plates  
containing KpnI/HindIII cut and SAPII  
pUC19 - but there are numerous white colonies  
on the 'test' plates and some on the  
CM<sup>+</sup> plates.

Neil's competent cells are actually better than the ones supplied by In Vitrogen.

Prepared a 1% Agarose gel for analysis of PCR products - ran 5  $\mu$ l of 100  
alongside 1 Kb ladder.

Ordered 1 gm X-Gal from Amersham.

Checked PCR products from Neil's RNA - No product - indicating that my  
protocol is not useful in this case. Since Neil did get it to work. Will try his  
protocol when I get back / he gets back.

Set up 12 mini plates on potential ~~cat~~/resu clones - picked 4 from  
transformation using Invitrogen Competent cells; 4 from Neil's competent cells, and  
4 from those plated on chloramphenicol - Grown on master plates over at 37°C.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Sgt. W. K. Clarke*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

*David K. Clarke*

000154



Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

Mini-DNA Probs of Potential CAT/RSV clones.  
TITLE Virus harvest from Vero clone (5).

From Page No. \_\_\_\_\_

Started mini-preps -  $cm^A$  colonies grown in Amp<sup>R</sup> failed to grow - discarded. Proceeded with DNA extractions for the 8 remaining Amp<sup>R</sup> clones.

Checked CPE on Vero clone (5) infected with working stock. CPE is very advanced - will harvest later today and check yield the following week.

Set up KpnI digestions on  $\frac{1}{5}^m$  of Mini-prep DNA (extracted from 1.5 ml culture).

Did RXN in 30  $\mu$ l final vol. for 1 hr at 37°C.

Passaged Hep-2s at 1:10 and Vero at 1:20 so they should still be useable on Mon.

Phenol chloroform extracted KpnI digests and ethanol ppt<sup>ed</sup> - then set up.

Hind III digests and included RNase A (final vol. 30  $\mu$ l 1 hr 37°C).

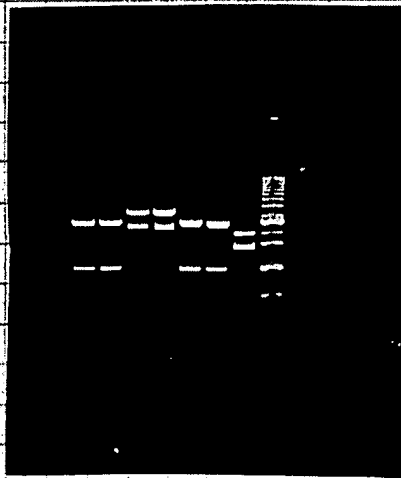
prepared a  $\frac{1}{2}$  Agarose gel and analysed final products.

14 15 16 13 11 12 10 / ladder (1)

(3) (4) (5) and (16) are it!

(1) probably is too.

Harvested Vero clone (5) cells infected with working stock. Staked at -80°C after freeze thaw (prior to end spin).



To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Sege W. Kibbe*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

*David K. Clarke*

TITLE:

CATCHING UP ON JOURNALS!

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

133

From Page No. \_\_\_\_\_

Checked growth of bacteria of CM<sup>+</sup> plates - No go - if the CAT gene is present (and it appears that it is) then it is not being expressed.

Copied a bunch of papers from Viro journals etc. for reading purposes.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kille*

Recorded by

*David K. Clarke*

000156

From Page No. \_\_\_\_\_

Took a look at passaged Vero/Hep-2 (from last week) - They appear to be okay, but will probably want and use the new cells

Digested plasmids 3, 4, 15, 16, with HgAI which should linearise the plasmids if they contain the correct insert

3  $\mu$ l x 10 buffer  
1  $\mu$ l plasmid  
1  $\mu$ l Enz  
16  $\mu$ l H<sub>2</sub>O + 1  $\mu$ l RNase A  
30  $\mu$ l. 1-2 hr at 37°C

Meanwhile prepared a 1% Agarose gel for analysis of digestion products

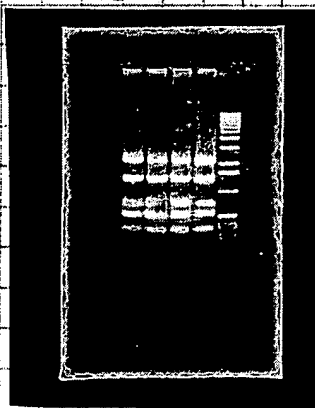
Ordered: Perkin Elmer RT-PCR kit

There are 5 DNA bands when the CAT/RSV construct is digested

1420\* = Key band containing CAT/RSV DNA  
747  
586 } probably migrate together  
560 }  
307

Set up 0/15 mini cultures of 4 and 16 (1  $\mu$ l Amp 50  $\mu$ g/ml)

Autoclaved



All requisite bands appear to be present in the correct position

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Steve W. Kibbe

Recorded by

David K Clarke

TITLE: Storage of CAT/RSV clones.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

135

From Page No. \_\_\_\_\_

Took off OAS cultures of CAT/RSV clones (4) and (16)  
Set up glycerol stocks of each culture.  
150µl of sterile glycerol  
850µl of bacterial culture.  
Vortexed briefly and stored at -80°C.

Prepared 2 x 500ml L-b broths - unlabelled

Prepared a stock of Amp 50mg/ml (filter sterilized - aliquotted and stored at -20°C)

Set up OAS plasmid cultures - added 500µl of Amp 50mg/ml to each flask + 500µl each OAS mini prep culture

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Steve W. Kuhl*

Recorded by

*David K. Clarke*

000158

From Page No. \_\_\_\_\_

Began plasmid preps - Spun bugs down 3.5K 30mins JA-14 rotor  
 Resuspended pellets in 10ml P1 (Qiagen series of solutions) - then added  
 10ml P2 (freshly made 0.2M NaOH 1/5 SDS) and incubated at R. Temp for 5-  
 10mins. Then added 10ml P3 - mixed thoroughly/gently and incubated on ice for  
 20mins - Spun in JA-20 for 30mins at 18K.

Pptd the super with 0.7 vols Isopropanol and immediately spun  
 at 10K for 45mins in the JA-20. - Rinsed off super and mixed pellet  
 with 70% EtOH (10ml) - re-pelleted 10mins.

Air dried pellet 10mins - then redissolved in 400µl T.E

buffer - retained 1µl for gel analysis and EtOH pptd the remainder - there  
 was quite a bit of chromosomal DNA present (fished out with a inoculat<sup>n</sup> loop)

Stored ppt in freezer in lab.

Ordered 293 cells from ATCC  
 (see opposite page)

Ordered T<sub>7</sub> Polymerase (5000 u)  
 from Promega

Deactivated  
 DNA from  
 NaOH treatment

There appears to be  $\approx$  500ng of  
 plasmid in  $\frac{1}{400}$ th of total

total  $\approx$  200000  $\approx$  200µg of  
 each plasmid in 1ml EtOH  
 $\approx$  200µg/µl in EtOH

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Invented by

Date

Sage W. Kuhl

Recorded by

David K. Clarke

293 cells

## .HEADLINE

ATCC CRL-1573 293 (Transformed primary embryonal kidney, human)

## .TEXT

Passage Frozen: 31. Current medium for propagation: Eagle's MEM with Earle's BSS, 90%; heat-inactivated horse serum, 10%. Additional Information: The 293 cell line is a permanent line of primary human embryonal kidney transformed by sheared human adenovirus type 5 (Ad 5) DNA. The cells are particularly sensitive to human adenovirus, are highly permissive for adenovirus DNA, and contain and express the transforming genes of Ad 5. Handle as potentially biohazardous material under at least Biosafety Level 2 containment. The line has been used in the isolation of transformation defective, host-range mutants of Ad 5, and is excellent for titrating human adenoviruses. This is a hypotriploid human cell line. The modal chromosome number was 64, occurring in 30% of cells. The rate of cells with higher ploidies was 4.2 %. The der(1)t(1;15) (q42;q13), der(19)t(3;19) (q12;q13), der(12)t(8;12) (q22;p13), and four other marker chromosomes were common to most cells. Five other markers occurred in some cells only. The marker der(1) and M8 (or Xq+) were often paired. There were four copies of N17 and N22. Noticeably in addition to three copies of X chromosomes, there were paired Xq+, and a single Xp+ in most cells. References: J. Gen. Virol. 36: 59-72, 1977; Virology 77: 319-329, 1977; *ibid.*, 86: 10-21, 1978. Submitted by: F.L. Graham, McMaster University, Hamilton, Ontario, Canada. Price Code: J

*Age W. Khl*

0000160

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE Checking large scale CAT/RSV plasmid Preps  
Control RT-PCR with Perkin Elmer Kit.

From Page No. \_\_\_\_\_

Spin down 5  $\mu$ l of each CAT/RSV plasmid ( $\approx 10 \mu$ g) - EtOH rinsed  
Dried and resuspended in 10  $\mu$ l  $H_2O$  (1  $\mu$ g  $\mu$ l<sup>-1</sup>) Then set up Kpn I digestion  
on 2  $\mu$ l

2  $\mu$ l DNA  
5  $\mu$ l x 10 buff.  
5  $\mu$ l x 10 BSA  
3  $\mu$ l  $H_2O$

Phenol chloroform extracted and EtOH pptd.

Proceeded by setting up Hind III digestion

3  $\mu$ l x 10 buff.  
20  $\mu$ l  $H_2O$   
1  $\mu$ l Enzyme  
3  $\mu$ l 1.5 hrs 37°C

Meanwhile prepared 1% Agarose  
gel and run digest

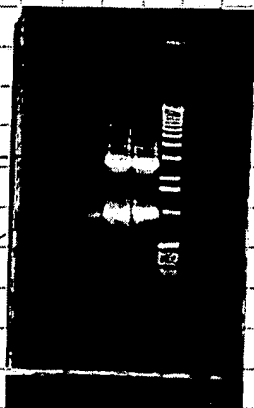
Perkin Elmer Cetus RT-PCR kit arrived - decided to set up control run - using  
Their control RNA + primers.

Set up reverse transcription of control RNA according to their protocol - after  
inactivation of RT at 99°C for 5 min. proceeded with PCR portion of control. (see protocol  
for details).

(Did not carry out  
initial 2 mins at 95°C)

Ordered  
DH5  $\alpha$  competent  
cells from BRL.

little bit of  
one-  
late bit  
of next plasmid.



Gel shows that the large scale plasmid  
preps of (+) (-) CAT/RSV are okay and  
giving the expected products following  
Kpn I/Hind III digestions.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sey W. Khe

Recorded by

Daniel K. Clarke

000161

Analysis of P/Eimer Control PCR RXNS

TITLE Preparation of RSV/CAT constructs for In Vitro

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

139

From Page No. \_\_\_\_\_

Transcription

Set up HgaI digestion on 8µg each of RSV/CAT (4) (16)

4.3µl H<sub>2</sub>O/DNA

5µl x10 buffer

2µl Enz

50µl

2hrs 37°C on at 9-30am

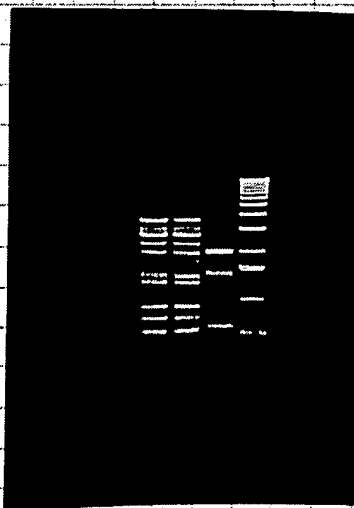
off 11-30am

Prepared Gel for analysis of PCR product (from 9µl rxn) and  
digest<sup>n</sup> products

Phenol Chloroform extracted HgaI digests and EtOH ppted

Set up Spectrophotometer (arrived in from Baxter)

It also appears that  
the control RT-PCR  
RXN did not work. Will  
set up again. This time  
used gloves and some of  
Neil's RNA and one rxn  
with Life Science R.T.  
also included a 60°C  
annealing step.



Gel shows incomplete HgaI digestion  
Will spin down and redigest for 2 hrs  
in 60µl using 3µl of enzyme - 2 hrs  
50 2

Will analyse on a gel  
tomorrow along with PCR  
products

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Serge W. Khle*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by

*David K. Clarke*

0000162



Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Checking Results of CAT/RSV (with HgA I)  
Checking PEmer Control RT-PCR RXNS

From Page No. \_\_\_\_\_

Prepared a 1% agarose gel for analysis of O/N PCR reactions

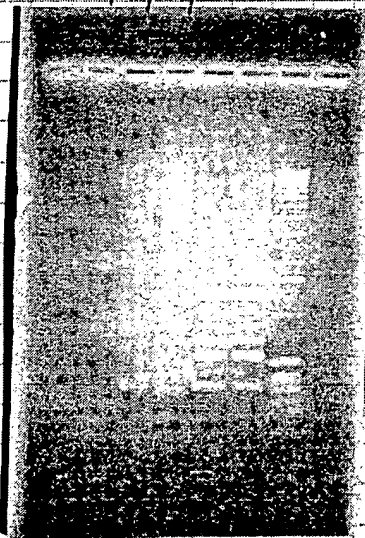
- ① Using Perkin Elmer RTase
- ② Using Life Science RTase
- ③ Using Neils Control RNA (from <sup>his</sup> PEmer kit)

Also checked reagents of CAT/RSV ④ ⑤ (with HgA I)

④ ⑤  
Life Science  
Myo  
Neils Control

At least 2 of the PCR  
RXNs have worked  
10-20 ng of DNA  
= 100-500 ng of total  
product.

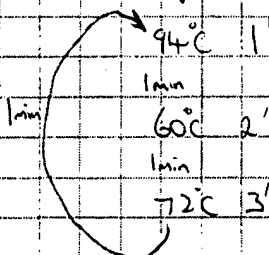
Life Science RTase did not  
give a product under these  
conditions.



Looks as though ④ ⑤ have  
cut further with HgA I - but  
still not completely - will order  
a fresh batch of Enzyme

The enz. conc. is actually  
v. low - explains why  
cutting is incomplete

PCR CONDITIONS were 2' 95°C → Ice prior to Tag addition  
Tag added (1/2 ml) overlaid with min. oil



Done's  
Will try Neils RNA Next

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne W. Kuhl

Recorded by

David K. Clarke

000163

Recutting CAT/RSV for 3rd Time!  
TITLE Setting up More RT/PCR Controls

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

141

From Page No. \_\_\_\_\_

Recut RSV/CAT (4/16) - boosted vol to 80 $\mu$ l and added 4 $\mu$ l more of H<sub>2</sub>O<sub>2</sub> - incubated at 37°C for 2 hrs until - then analysed 4 $\mu$ l on a 1% Agarose gel - Phenol Chloroform extracted and EtOH ppt'd

Infected Vero cells (25cm<sup>2</sup>) with 10<sup>-1</sup> and undil. B9320 - 0.25ml of each. Also infected 1x 25cm<sup>2</sup> with V<sub>3</sub> H<sub>2</sub> (32°C) - 0.25ml

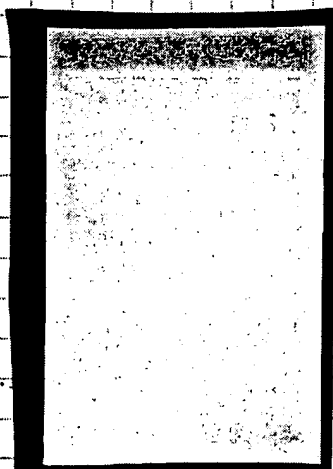
(Will grow Hep-virus at 32°C; Vero virus at 37°C)

Set up reverse transcription on X31 RNA (supplied by Dr. Stec)

Stuck to P. Elmer Protocol in general but with one difference.

Added 3 $\mu$ l (55) - Daves Primer to 5 $\mu$ l of RNA (24 $\mu$ g)  
Heated to 90°C for 1 min then snap cooled on ice - then added 3 $\mu$ l of this mixture to the RT mixture and incubated at 43°C for 1 hr 2-30 pm - 3-30 pm.

Good all the H<sub>2</sub>O<sub>2</sub> fragments are w they should be



200ng total in 4 $\mu$ l x 20  
= 4,000  $\approx$  4 $\mu$ g of cut plasmid

P.T.O

To Page No. \_\_\_\_\_

Witnessed & Understood by me.

Sgt. W. K. Kline

Date

Invented by

Recorded by

David K. Clarke

Date

0000164

From Page No. 141

After Reverse Transcription - heat denatured RT-ase at 80-90°C for 5 mins, Then added the PCR ingredients including 2µl of ssb. After mixing all ingredients did another 95°C treatment for a few mins to fully denature the DNA/RNA hybrids → Ice + 0.6µl Taq - added with mineral oil →

94°C 1min  
↓ 2min  
37°C 2min  
↓ 2mins  
72°C 3mins

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suz W. Khali

Recorded by

David R. Clarke

Checking PCR of Fin Rsv  
TITLE Harvest of  $V_3H_3$  32°C Virus.

Project No. \_\_\_\_\_  
Book No. \_\_\_\_\_

143

From Page No. \_\_\_\_\_

Prepared 1% Agarose gel, and loaded  $\frac{1}{10}$  (10  $\mu$ l) of ONV  
PCR reaction to see if 1.4 kb 'Fin' DNA was produced.

In the meantime checked c.p.e. on infected Vero/Hep-2 cells

Veros infected with  $V_2$  B9320 were not showing much c.p.e. - will check  
again this evening

The Hep-2s infected with  $V_3H_2$  were blown away! - Harvested  
and froze down as  $V_3H_3$  (32°C) Will titrate on Thurs

Oligos 155  
154

are the ones to be used  
to test the RT/PCR for  
RSV - set annealing  
at 37°C

2

D-1 155  
by  $\frac{1}{2}$

D-1 154 by  $\frac{1}{2}$ . Use 154 as cDNA primer



Good - the PCR RSV has  
worked giving a total of 2  $\mu$ g  
of product.

Dave's Oligos used were  
SS5 and SS6. They were  
approx. 30-mers with a m.p. of  
74°C. AT rich oligos.

Should probably set annealing temp  
for RSV work at 20-30° below  
m.p. (or 37°C) whichever is higher.  
also 2' between 94°C and annealing  
temp and 2' between annealing and  
72°C.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Sage W. Kuhl*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

*David R. Clarke*

000166

Checked c.p.e. on Vero cells - Undil shows good c.p.e. -  
strangely it had leaked (perhaps by capillary action). Replaced half of  
growth medium and continued incubation - will harvest this evening or  
tomorrow morning.

Set up a 2<sup>nd</sup> series of RT-PCR reactions

① Based on Methyl Mercury denaturation

② Based on no heat denaturation of RNA/cDNA (Just the usual 1' at 94°C during thermal cycling).

① Heat denatured RNA 90°C 2' → Ice

added 1µl of 10mM Methyl Mercury → 5mins RT

added 1µl of 1.85M B-Mercap → 5mins RT

added 3µl of ~~Dave~~ SS5 primer and then added 4µl total to

Reverse Tase mixture - 1µl of R-Tase → 42°C for 1.5hrs

② Heat denatured RNA + 3µl primer 90°C 2' → Ice

added 3µl of this mixture to R-Tase mixture - 1µl RTase → 42°C for 1.5hrs

Sege W. Kibiki

David K. Chashe

From Page No. 144

Set up PCR on ① after 5 mins at 90°C to inactivate reverse transcriptase.  
Used  $\approx$  1  $\mu$ l Taq. 1.5  $\mu$ l of 2<sup>nd</sup> (SSS) primer + additional  
 $\frac{1}{2}$   $\mu$ l of SSS

Phenol chloroform extracted the 'No heat' treatment ② - EtOH ppt'd with  
2  $\mu$ l of glycogen - will set up PCR with these products tomorrow.

Meanwhile harvested B9320 infected Vero  $\rightarrow$  V<sub>3</sub> B9320 dil/undil.  
Froze away at -80°C - will use to infect Vero tomorrow and do  
TCID<sub>50</sub>s.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sgt. W. Kibbi

Recorded by

David K. Clarke

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

TCID<sub>50</sub>s of B9320/Clone 5 Vero/V<sub>3</sub>H<sub>3</sub>

Analysis of PCR Products incorporating Methyl Mercury.

From Page No. \_\_\_\_\_

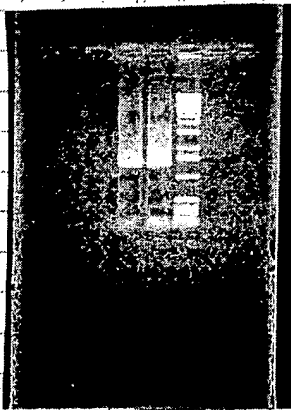
Prepared 1% Agarose gel for analysis of PCR products

LHS. loaded 1 Kb ladder / X31 / Methyl Merc. /  
(undil.)Infected Vero Cells with P<sub>3</sub> Vero B9320 → 10<sup>-1</sup> / undil.Carried out TCID<sub>50</sub> on - P<sub>3</sub> Vero B9320 10<sup>-1</sup> undil.

- Vero clone 5 progeny (infected with working stock)

- V<sub>3</sub>H<sub>3</sub>X31 Methyl Merc + heat  
denaturation

- X31 heat denaturation only



Products are essentially the same  
with or without methyl mercury - however  
this does not preclude that Methyl Merc.  
may be useful for V. long cDNA  
production.

Should try 1/10 dilution of this  
RNA = 2ng of template =

2 x 10<sup>4</sup> molecules

Set up PCR reaction with 'No Heat' sample

optimize annealing temp.  
+ elongation time  
heat denature?  
solvents?

Resuspended cDNA pellet in

70.4 µl H<sub>2</sub>O

10 µl x10 buff.

8 µl MgCl<sub>2</sub>

2 µl each dNTP

1 µl each primer

0.05 µl Taq

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Suzanne W. Kihl

Date

Invented by

Date

Recorded by

David K. Clarke

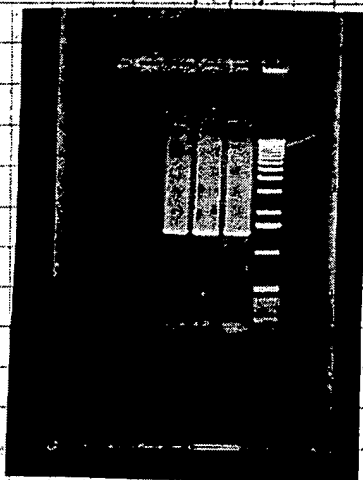
From Page No. \_\_\_\_\_

Prepared 1% Agarose gel for analysis of Q<sub>10</sub> PCR

Reaction (No heat used to initially denature RNA/cDNA hybrid). Will run

samples from 'X31' and 'Methyl Merc' R<sub>1</sub>X<sub>1</sub>s as controls.

Checked c.p.e. on B9320 infected cells - there are already some c.p.e. will probably be able to harvest tomorrow.



It appears that all of the conditions used were equally successful in producing a PCR product - will probably go with the Methyl mercury approach and a short 1" burn right before adding Tag.

Set up 4 Reverse Transcription reactions utilising the various

Nucleocapsid K<sup>+</sup> Tartrate / CsCl.

preparations

Vision.

12-14 / 12-27

Suspended RNA in 10 $\mu$ l H<sub>2</sub>O (DEPC treated) and 5 $\mu$ l of Oligo 154 (Invitrogen) - Heated to 90°C for 1'  $\rightarrow$  Ice - cooled for Methyl Mercury, followed by 1 $\mu$ l of 2-mercaptoethanol - Added 4 $\mu$ l of this mixture to the Reverse transcription mixture and 1 $\mu$ l RTase  $\rightarrow$  42°C for 1 hr

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Sgt W. Kibbi*

Date

Invented by

Date

Recorded by

*David X Clarke*



Project No. \_\_\_\_\_

Book No. \_\_\_\_\_ TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

*See reference books #22A*

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

From Page No. \_\_\_\_\_

Checked Infected Vero cells (B9320) - They have been  
'blown away' at  $10^{-1}$  and 'undil' - Harvested and froze down as  
B9320 P<sub>1</sub> Vero  $10^{-1}$ /undil

Checked 293 Cells - Many had settled down and were growing okay;  
Rinsed off the supernatant containing dead floating cells and re-fed  
with 5ml growth medium.

Prepared a 1% agarose gel and analysed 10  $\mu$ l samples of O/N  
PCR RXN



Fantastic - all the various RSV genome  
preps have given me a RT-PCR product  
of 550bp

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Steve W. Kibbe*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

*David K Clarke*

0000172

From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub>s - B9320 Virus appears to have grown  
v. well; about  $10^6$  ml<sup>-1</sup>

Vero clone (S) is also producing  $10^5$ - $10^6$  ml<sup>-1</sup> which is quite a good  
titer.

V<sub>3</sub>H<sub>3</sub> titer is much lower than expected - perhaps because

Virus set too long at 37°C when all the cells were wiped out (before harvest)  
or perhaps the virus is adapting to Hep-2s so well that they are not growing  
well as Vero (indicator cells!)

Will make final TCID<sub>50</sub> readings tomorrow

Checked 293 cells - they are growing fairly well (without horse serum)  $\approx 70\%$  confluent

\* Ordered Lipofectance Reagent (Inv) from BRL. 2<sup>nd</sup> day delivery

Infected Hep-2 (TC 150s) with P<sub>3</sub>Vero (working stock) - used 1 ml / flask

Infected Vero (TC 25s) with [P<sub>3</sub>V<sub>3</sub>9320] 10<sup>6</sup> / undil.

Adsorbed  $\approx 3$  hrs at 37°C

Will need oligo for making 1<sup>st</sup> RSV L clone  
(Heme class primer)

5' ACG/AGA/AAA/AAA/GTG/TCA/A

This clone will be  $\approx 3$  Kb (2971) if it works out

19-Mar

M.p. = 50°C

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge W. Kuhl

Recorded by

David K. Clarke

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

151

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

Will use some of Vernon RAA/Pinder 154 (already at  $-20^{\circ}\text{C}$ ) and some  
fresh FROH ppt from  $\text{K}^+$  tartrate

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sgt W. Kuhl*

Recorded by

*David F. Clarke*

000174

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

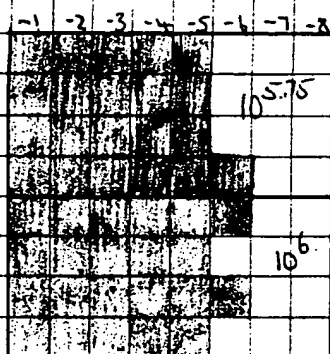
TITLE

TCID<sub>50</sub>s.

In Vitro transcription of CAT/RSV.

From Page No. \_\_\_\_\_

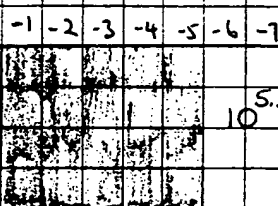
Checked infected vero cells - no signs of c.p.e yet - will check again later this afternoon. Hep-2s are showing a few syncytia - should be okay by tomorrow.

10<sup>5.75</sup>

89320

P<sub>3</sub>10<sup>-1</sup> unit.10<sup>6</sup>

Veroil (cf.)

10<sup>5.5</sup>V<sub>3</sub> H<sub>3</sub>  
unit.10<sup>6</sup>

Vero Clone 15

P<sub>1</sub>V (infected with working stock)

293 cells are looking pretty good  $\approx$  80-90% confluent.

Set up In Vitro transcription of CAT/RSV (H<sub>3</sub>A<sub>3</sub>) digested (f) and (16)

4 $\mu$ l x5 Rn buffer2 $\mu$ l 100mM DTT2 $\mu$ l RNasin4 $\mu$ l rNTPs1 $\mu$ l DNA Template (2 $\mu$ g)7.5 $\mu$ l H<sub>2</sub>O20 $\mu$ l final Vol.+ 1 $\mu$ l of T<sub>7</sub> RNA

off at 4:20 pm

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suz W. Kiehl

Recorded by

David K. Clarke

0000175

Gel analysis of IVT CAT/RSV RNA.  
Virus Harvest. P<sub>3</sub> V.P. H-2.

Project No. \_\_\_\_\_

TITLE \_\_\_\_\_

Book No. \_\_\_\_\_

153

From Page No. \_\_\_\_\_

Checked infected Vero's - They are not showing v. advanced c.p.e. - perhaps last hit in previous infection (since 10<sup>-1</sup> is not showing good c.p.e. either it probably is not due to DI buildup. Will leave until tomorrow to harvest. Will want to titrate P<sub>4</sub>V and P<sub>5</sub>V B9320 before deciding on any further infections.

Checked HEP-2s - There are quite a few syncytia - will harvest later today

Prepared 1% MOPS Agarose gel with formaldehyde

5ml x 10 MOPS

37ml H<sub>2</sub>O

8 ml Formaldehyde

Took 4  $\mu$ l aliquots from IVTs 4/16 - added to 10  $\mu$ l denat. formamide - 10  $\mu$ l

X1 MOPS gel buffer - added 1  $\mu$ l of 1mg/ml<sup>1</sup> EtBr - heated to 65°C for 5 mins - added 2  $\mu$ l of RPB and loaded on gel with 1.5  $\mu$ l of

RNA markers

1. Checked 293 cells - They look reasonable, but will require a couple more days before they are fully confluent.

Harvested V<sub>3</sub>H<sub>1</sub> virus (48 h p.i.)  
There were quite a few syncytia  
Stored frozen as V<sub>3</sub>H<sub>1</sub> 32°C

Also shifted Vero infected with B9320 (until) to 32°C (left 10<sup>-1</sup> at 37°C)

Seems to be the correct size of transcript for CAT/RSV.

1,892 marker.  
872 marker.

Witnessed & Understood by me,

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

*Sey W. Kuhl*

Recorded by \_\_\_\_\_

*David R. Clarke*

To Page No. \_\_\_\_\_

000176

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

More In Vitro Transcription of CAT/RSV.  
TITLE Gel Analysis of Transcripts

From Page No. \_\_\_\_\_

Checked 293 Cells - they are not growing quickly - will take at least until tomorrow to achieve 80-90% confluence.

Set up In Vitro Transcription of CAT/RSV

Meanwhile prepared  
1% Mops/Agarose Gel.

8  $\mu$ l x 5 buffer

4  $\mu$ l 100mM DTT

1  $\mu$ l RNasin

8  $\mu$ l rNTPs 2.5mM each

2  $\mu$ l DNA Template

15  $\mu$ l H<sub>2</sub>O

2  $\mu$ l T<sub>7</sub> Pol

40  $\mu$ l

1 hr 37°C

Loaded Gel: LHS / RNA Markers / IVT / IVT / Viron / K-Tert  
1.5  $\mu$ l 4 16 16 16 16  
4 4 4 4

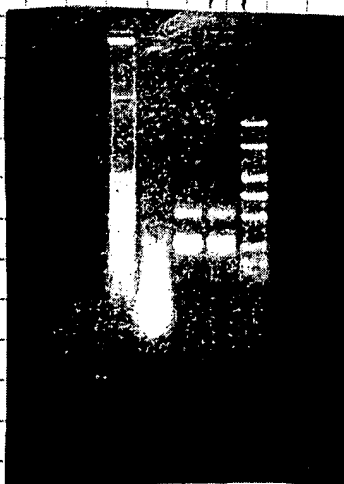
Harvested V<sub>5</sub> B9320 10<sup>4</sup>/undil - Will do TCED soon this afternoon.

IVT (1)  
IVT (2)

In Vitro Transcription looks  
V<sub>5</sub> good.

It seems there is 0.5-1  $\mu$ g  
of RNA transcription

11500 4  $\mu$ l of IVT mix  
200 ng/band Will use 10  $\mu$ l  
for Transfection



15K?  
-9400 } 3263  
-525 } 2314

Gave Kim Vero  
Clone (5) for  
growth and testing

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Steve W. Kibbe

Recorded by

David K. Clarke

TITLE: TCID<sub>50</sub>

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

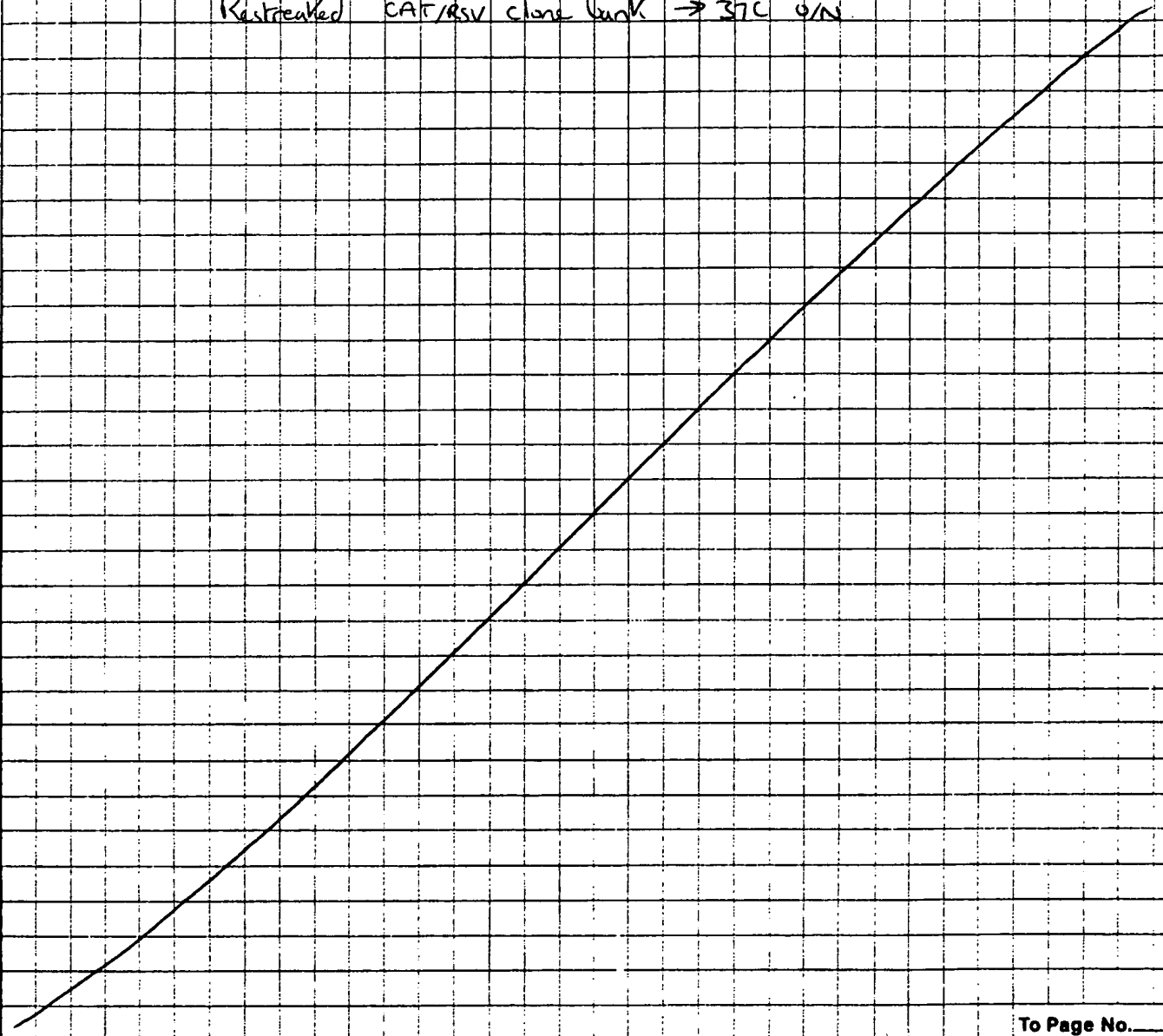
155

From Page No. 154

Carried out TCID<sub>50</sub> on  $\left. \begin{array}{l} P_1 \text{ Vero 89320} \\ P_3 \text{ Vero 89320} \end{array} \right\} 10^{-1} / \text{undil.}$   
 $\frac{1}{3} H_1 \text{ (Stock) } 300 \mu\text{C.}$

Diluted Oligo (Rsv 5' to Pmt) to 100 ng  $\mu\text{L}^{-1}$  (working stock).

Restrained CAT/RSV clone bank  $\rightarrow 37^\circ\text{C}$  O/N



To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Sege W. Kelle*

Date

Invented by

Date

Recorded by

*David R. Klatte*

000178



From Page No. \_\_\_\_\_

Checked 293 cells - they look pretty good - some parts of the monolayer are a little sparse, but others are showing higher density

Resuspended clones are not growing vigorously - will resuspend again on a fresh Amp<sup>r</sup> plate, and leave at 37°C exp again to boost growth.

Went over CAT Assay with Pamuk.

### Protocol for Transfection of CAT/RSV Transcripts

- 1) Infect  $\approx 100$  confluent 293 cells (in 6 well plate) with RSV with as high a M.O.I. as possible - Consider  $10^6$  293 cells per 6 well plate - will use  $\frac{1}{2}$  ml of highest titer virus stock ( $\approx 10^7$  ml<sup>-1</sup>) to give an M.O.I. of  $\approx 5$ /cell.  $\rightarrow 37^\circ\text{C}$
  - 2)  $\approx 1$  hr after putting on virus remove inoculum - rinse cells x 2 with Opti-MEM reduced serum medium
  - 3) Add transfection mixture (1ml) and incubate cells for 3hr  $\rightarrow$  5hr at  $37^\circ\text{C}$   
(Can leave on life this)
  - 4) Then add 1ml of DMEM containing 1% serum (not really necessary)
  - 5) Retain some of the supernatant (add a little  $\text{MgSO}_4$  and Hepes) for possible passage
- Set up reverse transcription/PCR as before - extended polymerization cycle to 5min and used RSV's for PstI primers.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge W. Kholi

Recorded by

David K Clarke

TITLE Analysis of RT/PCR products

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

157

From Page No. \_\_\_\_\_

Prepared 1% gel for analysis of RT/PCR products  
loaded 10  $\mu$ l of 'Viron', 'Nucleocapsid - K<sup>+</sup> test.' and 1  $\mu$ l of  
IKB ladder

LHS / IKB ladder / Viron / Nucap

Stored Bacterial clones at 4°C - the plates are too thin to support  
vigorous growth - will restreak again early next week, on my own plates.



Results look v. encouraging. It now  
seems possible that long PCR products  
are possible - perhaps DM50 may increase  
yield. - Could also use more template

Will electroporate purify and clone

Future experiments

- 1) Test Monoclonal antibody efficiency of neutralisation of  
B7320. (Plaque/TCID<sub>50</sub> reduction titre)
- 2) Transfect in K<sup>+</sup> testate gradient genomic RNA.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sgt. W. Khl*

Recorded by

*David K. Clarke*

0000180

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

TCID<sub>50</sub> reading.  
Infection of Hep-2s for N.capsid.  
Transfection of 293s. with CAT/RSV RNA.

From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub> - V<sub>3</sub>H<sub>2</sub> show a titer of  $\approx 10^{6.5}$  - good - this will be an ideal stock for infecting Hep-2s for N.capsid.

TCID<sub>50</sub> of V<sub>3</sub>H<sub>2</sub>/V<sub>5</sub> B9320 Dil/unit - indicates that should use  $10^4$ /unit of " $10^4$ " V<sub>5</sub>-B9320 for next infection - will show final read out tomorrow.

293 cells look pretty good.

Infected Hep-2 cells (225cm<sup>2</sup> monolayers) each with 1.5ml of V<sub>3</sub>H<sub>2</sub> (from 1-19-94) - adsorbed 3hrs at 37°C then fed with 20ml of MEM and incubated 48hrs at 32°C. 10am - 1pm.

Ordered Oligos Bam HI to Pml  
and Pml to Bam HI.

m.p 50°C  
18mer

Bam to Pml

5' GAG / AAA / ATG / GAT / CCC / ATT

m.p 50°C  
17mer

Pml to Bam

5' CTC / ACC / ACG / TGT / TAA / AC

Used V<sub>3</sub>H<sub>2</sub> 32°C. to infect 293 cells ( $10^7$  phn/ml)  $\approx 0.5$  ml / small plate  
(mixed monolayers 1st with PBS - should use prewarmed medium).  
adsorbed for 1hr at 37°C with occasional gentle shaking.

Meanwhile prepared Optuscan/Transfectance mixture 90µl: 12 µl resp.  
Then prepared Rmt/optimum mixture 10µl: 90µl resp.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suz W. Kuhl

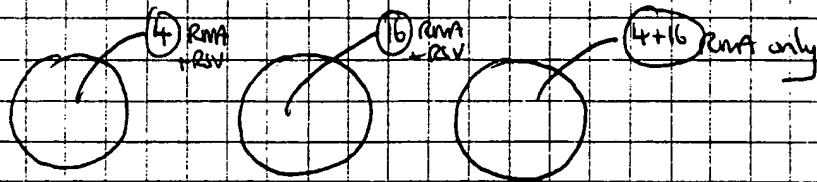
Recorded by

David K. Clarke

TITLE: \_\_\_\_\_

From Page No. 158.

Then at a suitable time point mixed RNA/optimer with Transfactor/optimer  
let sit at room temp for 15 min, then diluted with 0.8ml of  
Optimer and added to monolayers which had been pre-rinsed x2 with  
Optimer - left o/n at 37°C.



To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sege W. Khlle*

Recorded by

*David K. Clarke*

000182

From Page No. \_\_\_\_\_

50%  
30%  
Checked c. pe. on infected Hep's / 293 cells -

Did final reading on TCID<sub>50</sub>s

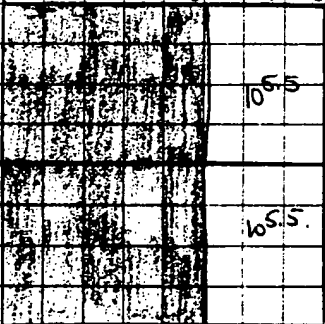
293 cells look v. sick - especially those which have been infected with RSV - will harvest this afternoon.

-1 -2 -3 -4 -5 -6

10<sup>6.5</sup>V<sub>3</sub>H, 32°C

Good yield - will use this stock to prepare another batch of nucleocapsid i.e. can use as working stock.

-1 -2 -3 -4 -5 -6 -7 -8

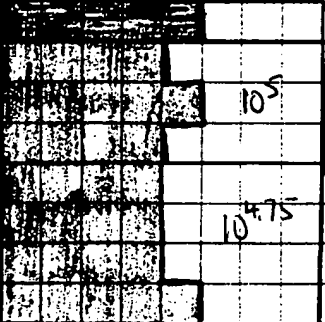
10<sup>5.5</sup>V<sub>5</sub> B932010<sup>-1</sup>

B virus is growing fairly well - will use

"10<sup>-1</sup>" stock for P<sub>1</sub> passage to see if

can assist titer any more - perhaps can move into Hep's.

-1 -2 -3 -4 -5 -6 -7 -8

10<sup>5</sup>V<sub>4</sub> B932010<sup>-1</sup>

Titers may be affected by virus 1/2 life at 32°C - not good to leave once c. pe. is

v. advanced - may have happened here.

10<sup>4.75</sup>

Undil.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge W. Kibbe

Recorded by

David K. Clarke

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

Will order 1 small Hep + 2 small Venus for next mon.

Need to set up Plaque reduction assay B9320/AR using Monoclonal antibody in preparation for genome rescue using B9320 as helper.

3pm initiated harvest of CAT Extract.

- ① Aspirated off supernatants  $\rightarrow$  froze in EtOH/dry ice
- ② Rinsed x 2 with 2ml of cold PBS
- ③ Added 1ml of xITEM and resuspended cells; by scraping etc.
- ④ Spun at 3K r.p.m for 1 min at 4°C in eppendorf
- ⑤ Resuspended pellets in 100 $\mu$ l of 0.25M Tris pH 7.5 ice-cold
- ⑥ went through x3 freeze thaw cycles - Dry ice/EtOH  $\leftrightarrow$  37°C
- ⑦ Spin down debris 5 mins, 14K eppendorf 4°C - collected super and freeze rapidly in EtOH/dry ice

Checked c.p.e. on Hepa - already starting to develop fairly well.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sege W. Kuhl*

Recorded by

*David K Clarke*

000184

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE RSV Nucleocapsid prep.  
CAT Assay of transfected 293 cells.

From Page No. \_\_\_\_\_

Prepared 100ml of L-Agar - added Amp and poured plates  
for restreaking of clone banks.

Inoculated Nucleocapsid prep in 10x TC 225s which were  
showing advanced c.p.e. (fusion).

Followed Extraction protocol carefully - used 2.10ml of lysis buffer  
per 5x TC 225s.

prepare 6  $K^+$  tartrate gradients 55% (w/w)  $\rightarrow$  15% (w/w).

Used 800 $\mu$ l of each conc, but only 200 $\mu$ l of 15% since  
nucleocapsid was resuspended in 15%  $K^+$  tartrate.

Meanwhile restreaked CAT/RSV clones on Amp<sup>r</sup> L-agar plates.

Also set up CAT assays using 20 $\mu$ l of Transfected cell extract.

2 $\mu$ l  $^{14}C$ -chloramphenicol (identically 200 $\mu$ l ml<sup>-1</sup>).

20 $\mu$ l 40mM Acetyl coA.

in 10s - Cl pH 7.5.

See protocol - Run for 3hrs at 37 $^{\circ}C \rightarrow$  -70 $^{\circ}C$  over

Put on  $K^+$  tartrate spin at 6pm - should be off at 10am.

Diluted Oligos Pml to Bam and Bam to Pml to 100 $\mu$ g ml<sup>-1</sup>  
Stored at -20 $^{\circ}C$  - stored.

Ordered Trial Oligo from "Oligos etc."

5-GGG/GCA/AAT/ACA/AAG/ATG/GCT/CTT/AGC/  
AAA/GTC/AAG/TTG/AAT/GAT/ACA/CT -

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Seyi W. Kille

Recorded by

David K Clarke

Running of CAT TLC.

Harvesting Nucleocapsid from gradient. + further purification of virus genome

Project No. \_\_\_\_\_

TITLE: \_\_\_\_\_

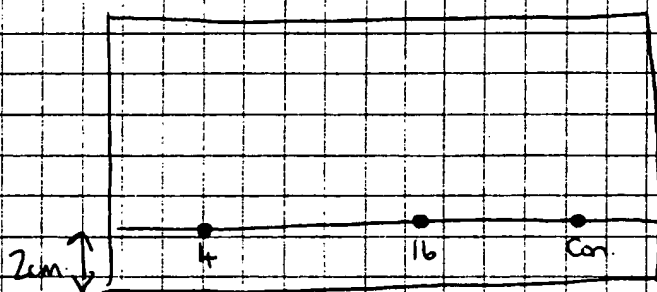
Book No. \_\_\_\_\_

163

From Page No. \_\_\_\_\_

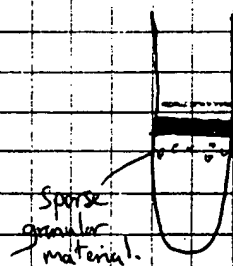
Ethyl acetate extracted CAT RNAs - used 1ml with 3x 10 sec pulses of vortexing for each - collected ethyl acetate and speed varied down - then resuspended in 30ul ethyl acetate prior to dotting on TLC.

Meanwhile equilibrated chromatography tank with ~~190ml~~ 190ml Chloroform 190ml of Methanol. Dotted on resuspended samples - using a capillary tube.



Let TLC go for 2 hrs (2pm off)

Realised that I screwed up Nucleosid prep (forgot to do nucleus spin)



Potential Nucleosid band.  $\approx \frac{1}{2}$  way down gradient.

Dense band of something - probably cellular DNA etc.

Harvested different fractions and tried to pellet Nucleosid band, by dilution into SW 50.1 tubes and spinning at 35K for 1hr 50c

Also tried SDS proteinase K digestion of thick band of material - seemed v. resistant to digestion

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Suzanne K. Kibbe*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by

*David K. Cluthe*

UUUU186



From Page No. 163

Resuspended potential nucleocapsid in H<sub>2</sub>O and SDS/protease K  
treated for 1 hr at 57°C - then phenol chloroform extracted and EtOH ppt'd.

Somehow didn't look quite right.

Completed TLC, and put away for autoradiography.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Serge W. Koble*

Recorded by

*David F. Clarke*

000187

Checking N.capsid prep for presence of genomic RNA.

Project No. \_\_\_\_\_

TITLE \_\_\_\_\_

Book No. \_\_\_\_\_

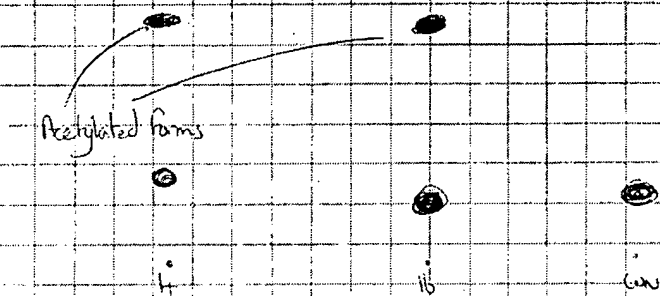
165

From Page No. \_\_\_\_\_

Developed CAT/RSV rescue autograd - Safe light was not safe !!

However can see that the rescue worked - re-exposed autograd - will

develop tomorrow.



Ran out 3kb PCR products Virus/Nucleocapsid on a 1% agarose -  
purified onto NA+5 paper and eluted into high salt buffer - phenol chloroform  
extracted diluted x2 with H<sub>2</sub>O and EtOH ppt'd with 10mg glycogen.

Proceeded to set up DNA ligation with T/A cloning vector (Assume 45ng for Virus  
PCR product)

2ul x10 buffer

2ul x10 buffer

2ul Vector

2ul Vector

3ul insert 50ng (50ng of 10)

DNA insert in 15ul H<sub>2</sub>O (All of 3kb Virus) 12°C /ON.

450ng x 2 = 225ng for  
Nucleocapsid PCR  
product

1ul T/A DNA ligase

1ul T/A DNA ligase

Also Ran a MOPS/Agarose gel to analyse potential Nucleocapsid preps

Neither prep looks promising



Stained material does not seem  
to be in the correct position  
for genomic RNA

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Serge W. Kuhl*

Date

Invented by

Date

Recorded by

*David K. Clarke*

0000188

From Page No. \_\_\_\_\_

Set up ligation of 3Kb RT/PCR product - electro-elute purified on NAKT paper from yesterday

3Kb from Ncap	3Kb from Vector	Control
2 $\mu$ l x10 lig <sup>n</sup> buffer	"	"
2 $\mu$ l Vector (50ng total)	"	"
5 $\mu$ l Invert DNA (50ng)	1.5 $\mu$ l Invert (50ng)	1.5 $\mu$ l H <sub>2</sub> O
10 $\mu$ l H <sub>2</sub> O	Used total amount due to too yield.	
1 $\mu$ l T <sub>4</sub> ligase	1 $\mu$ l T <sub>4</sub> lig	1 $\mu$ l T <sub>4</sub> lig
20 $\mu$ l	"	"

Stored remaining  
1.5 $\mu$ l at  
-10 $^{\circ}$ C

Left qn at 12 $^{\circ}$ C

Redeveloped CAT/RSV Rescue TLC - looks v. good Both

④ and ⑫ constructs are giving positives - CON is Neg.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sgt. W. K. Kline*

Recorded by

*David K. Clarke*

TITLE: Bacterial Transformation with Bstential JK viral sequence

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

167

From Page No. \_\_\_\_\_

Carried out Bacterial transformation - used 3 set of different competent cells.

1) Neil's

2) Invitrogen T/A system.

3) BRL Transfinity

Added 2 $\mu$ l of each ligation reaction (con, Ncap. Virus) to the BRL Transfinity; used 5 $\mu$ l from each ligation mixture for the other Bacterial cells.

Heat shocked BRL/Neil's cells for 1min at 42°C  
T/A cells 30secs at 42°C

Added SOC and incubated at 37°C for 1hr - then plated out 200 $\mu$ l from each mixture  $\rightarrow$  O/N 37°C

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Sege W. Kulu

Date \_\_\_\_\_

Invented by \_\_\_\_\_

recorded by \_\_\_\_\_

David K Clarke

Date \_\_\_\_\_

0000190

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Science Meeting

Potential 3K clones on Master Plates

B9320 grown on Hep 2

From Page No. \_\_\_\_\_

am Science Review

Took transplants from yesterday's experiment from the incubator and put at 4°C.

Infected Hep-2 cells with "B9320 V<sub>5</sub> 10<sup>-1</sup>" 0.25 ml / 25 cm<sup>2</sup> Flask

Also infected Vero with the same stock - until/dil.

Meanwhile streaked out Potential 3K clones on Amp<sup>r</sup> Master plates

Selected 10 clones each from Neupend/Viron T/A Exp  
 5 clones each " " " Neil's bridge  
 5 " " " " " BRW bridge

at 506  
 Trail  
 per

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Lyle W. Kuhl

Recorded by

David K. Clarke

000191

TITLE:

Passage of CAT/RSV supernatant from  
transfection

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

169

From Page No. \_\_\_\_\_

checked c.p.e. of B9320 on Hep-2s and Vero.  
Vero c.p.e. is extensive on uninfected

11-22-93 (P3 V)

pm Infected 293 cells with "working stock" to check if they produce

More virus - also passaged supernatant from ~~transfected~~ CAT/RSV  
rescue Exp (Added 1/2 ml of transfection super to 0.25 ml of helper - high  
later stuff) ~~V<sub>3</sub> H<sub>2</sub> 32°C~~ V<sub>3</sub> H<sub>1</sub> 32°C

Set up O/N mini preps on 8 potential 3kb clones - 100 µg/ml Amp

Selected clones	Virus (V)	T/A 1-5	NC	T/A 1-5
"	"	Nels 13+14	"	Nels 13+14
Virus	"	BRL 19+20	"	BRL 19+20

Harvested. Virus infected Hep-2s - (B9320 V<sub>5</sub> H<sub>1</sub>)<sup>uninf</sup>  
Vero - B9320 V<sub>6</sub> D1.  
" " " uninf. (extensive c.p.e.)

Phone calls out to potential R/As!

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sage W. Kibbe

Recorded by

David K. Clarke

000192

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE \_\_\_\_\_

Harvest of CAT/RSV DI from Passage of  
Supernatant from Infection Exp.

Mini-preps of potential 3K clones.

From Page No. \_\_\_\_\_

Carried out mini preps on the 18 potential 3K clones —  
went through procedure and stored DNA after EtOH ppt — dried  
pellet and stored at  $-20^{\circ}\text{C}$  — will sea I digest etc tomorrow — will  
use  $\frac{1}{2}$  of total.

Also harvested <sup>293</sup> cell extract which had been produced from infection  
with rescued RSV/CAT/Helper ( $\approx 200$  p.i.) — freeze away at  $-80^{\circ}\text{C}$ .  
Also kept 1ml of supernatant for further passage.

3pm left for Dentist to have a tooth taken out!!

I don't think I'll be back today.

Will Run HI/HOI on mini-preps — save 10 $\mu$ l of 50 for gel  
then sea I out  $\approx$   $\left. \begin{array}{l} 1400 \\ \sim 900 \\ \sim 700 \end{array} \right\}$  frags

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Sage W. Kibbe

Recorded by

David K. Clarke

Mini-prep analysis for 3Kb insert.  
TITLE: B9320 TCID<sub>50</sub>

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

171

From Page No. \_\_\_\_\_

Checked infected 293 cells - they are showing v. nice signs of  
c.p.e. - fusion, giant cells etc - will harvest later today  
Will set up <sup>XhoI</sup> BamHI digest first (lower salt rxn) on 1/2 of total  
mini prep DNA prepared yesterday

Did BamHI cuts in Sopl with 1  $\mu$ l (20  $\mu$ l) of Enzyme  
Gave 1. Shrs at 37°C - then  $\phi$  chloroform extracted and ppt'd at  
-80°C prior to XhoI digest - saved a little after double digest for analysis.

Carried out TCID<sub>50</sub> titrations on B9320 V<sub>5</sub> H<sub>1</sub> (uninfected); also  
B9320 V<sub>6</sub> 10<sup>-4</sup> and V<sub>6</sub> undil.

Sca I also cuts T/A vector to give (from m.c.s.) fragments  
2113 and 1819.

MON  
642-5240  
\$175  
10-30 - 3-30

Set up RT/PCR rxn on NCapsid RNA (thml spun down) - resuspended  
in 3  $\mu$ l H<sub>2</sub>O 3  $\mu$ l Bam  $\Rightarrow$  Pml primer (100 ng  $\mu$ l<sup>-1</sup>) - heated at 92°C for  
2 mins  $\Rightarrow$  Ice (1/2  $\mu$ l Meth Merc - 5 mins RT - 1/2  $\mu$ l 2-mercapto-  
5 mins R.T.) Used 4  $\mu$ l of this mixture in

standard Perkin Elmer Rev Trans<sup>TM</sup> RXN followed by PCR  
Extended program 44 72°C permut to 7 mins 30 cycles

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Sgt. W. K. Kile

Date

Invented by

Recorded by

David K. Clarke

Date

0000194





**TITLE**

Further Mini-prep analysis for 3Kb. insert...

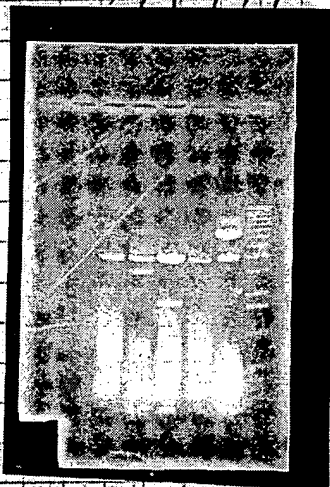
### Analysis of Long PCR RXN.

**Project No.**

Book No.

**From Page No.\_\_\_\_**

Prepared 2 x 1% Agarose gels for analysis of remaining BamHI/XhoI cut mini prep DNAs. Also loaded 10  $\mu$ l (of 100) from O.N. RT/PCR rxn, to check for potential 3.8 kb product from nucleocapsid prep?

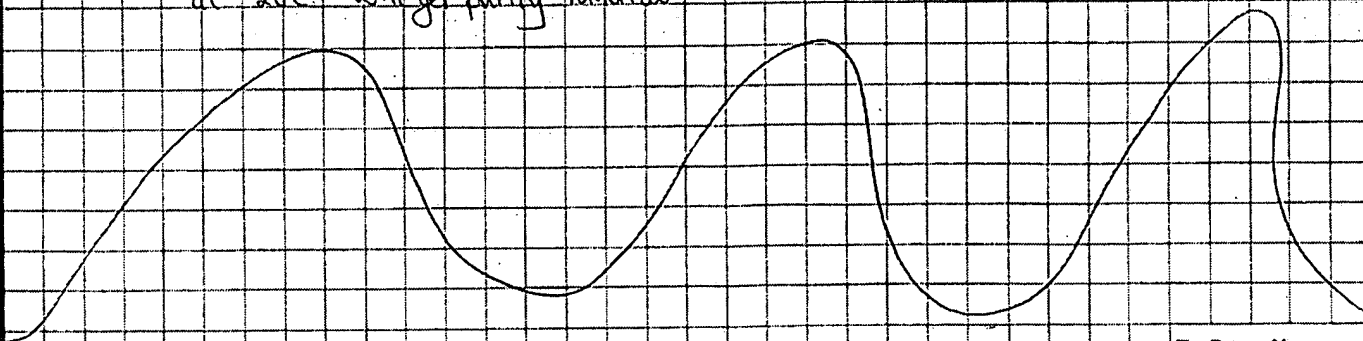


results look

V. encouraging -  
looks as though I  
have many correct  
3rd classes - will  
complete Seal digest  
analysis on Mon.

Also 3.8 kb PCR product looks pretty good and should yield clones

Chloroform extracted 3.8Kb RT/PCR mixture and EtOH ppt'd 0.15  
at -20°C. Will gel purify tomorrow.



To Page No

**Witnessed & Understood by me,**

Sege W. Kuku

**Date****Invented by**

Date \_\_\_\_\_

Recorded by

ded by  
David K Clarke

000196

From Page No. \_\_\_\_\_

prepared 1% Agarose gel for Electrophoresis of 3.8Kb fragment.

Spun down RT/PCR mixture from yesterday - resuspended pellet in 15  $\mu$ l x1

Electrophoresis buffer and loaded on gel with marker ladder.

Designed primers for ~~RT/PCR~~ <sup>HK</sup> RT/PCR product  $\rightarrow$  " "

XhoI  $\rightarrow$  BamHI  $\rightarrow$  BamHI mp = 54°C

cDNA primer 5' TAC CAA GAG CTC GAG TCA

Bam  $\rightarrow$  XhoI  $\rightarrow$  XhoI mp = 54°C

5' AAT GGG ATC CAT TTT GTC C

The appropriate 3.8Kb DNA band (x 50-100  $\mu$ g) was eluted onto NA45 paper, rinsed briefly with NET buffer and collected in High salt NET (100  $\mu$ l - 2x 50  $\mu$ l rinses) - This was diluted x2 and mixed with 7  $\mu$ l of glycogen, then EtOH potted on at -20°C.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kibbe*

Recorded by

*David K. Clarke*

000197

Infection of TC 225s for N. capsid prep.  
TITLE: Sca I digest<sup>n</sup> of potential 3K clones

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

175

From Page No. \_\_\_\_\_

Ordered Oligos (see yesterday) from Ed - "No waiting" so should have them by Wed/Thurs

Checked TCID<sub>50</sub>s - Used wrong TCID<sub>50</sub> plates (cells were not confluent) - Other plate is fine - will repeat the entire series on Wed.

Infected 10 x TC 225. (11-45am) - each with 1.5m of "V<sub>3</sub>H, 32°C" adsorbed 3hrs at 37°C then switched to 32°C.

Set up Sca I digests of Bam HI/Xho I digested mini preps

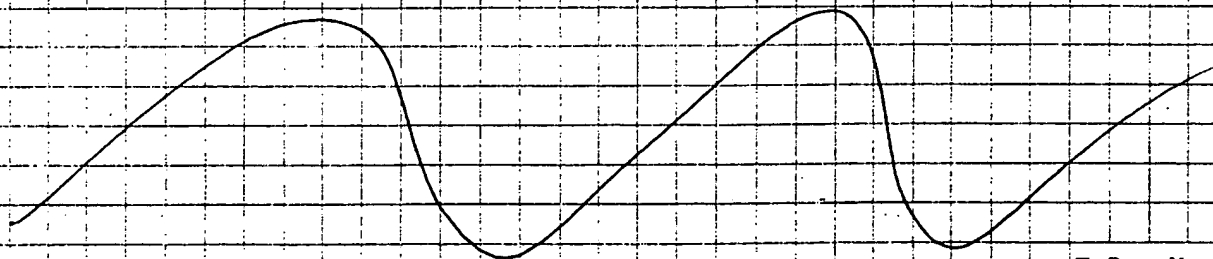
Digested only those which appeared to have correct insert. Would

predict the following bands

2,113	} from PCR II Vector which has
1819	
~14000	} from 3Kb insert
~9000	
~700	

2-3hrs at 37°C. 30µl final vol 10µl (10m) enzyme

Carried out CAT assay on 20µl of cell extract from the passage of yield from the 1<sup>st</sup> transfection experiment. - let it run for ~ 2 1/2 hrs at 37°C - Then freeze down until tomorrow



To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Dr. V. K. K. K.

Recorded by

David K. Clarke

0000198

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE \_\_\_\_\_

Exp. design for differentiating between  
mixed Subtype A and B RSV populations

From Page No. \_\_\_\_\_

 $\sim 10^6$  89320

+

10, 100, 1000 'A' virus

mixed with conc. monoclonal antibody(s)  
and incubated at 37°C for 2 hrsNeed to establish plaque  
efficiency of RSV 1stPlate out total on 150cm<sup>2</sup> Flask under  
1 to 3 diluted neutralizing monoclonals

Pick large selection of plaques

24 well plates in duplicate (Vero)

24 h. p.i. add differentiating monoclonals - then  
add Rabbit-anti-mouse conjugated with enzyme

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Shirley W. Koble

Recorded by

David K. Clarke

Analysis of ScaI cut Mini preps.  
CR assay of passaged supernatants.

Book No. \_\_\_\_\_

From Page No.\_\_\_\_

Prepared 2 x 1% agarose gels - for analysis of Sca I digests.

Checked infected TC 225s (Heps) - they are not showing v. significant c.p.e yet - will check again this afternoon  $\approx$  5pm.

Loaded and ran agarose gels

Comed out ethyl acetate extraction of CAT assay products.  
Dried down in a speed-vac. - Meanwhile equilibrated the TLC tank  
with chloroform (190 ml) : methanol (10 ml)

✓ NE14

E14.

C B R L A

C

GR 10  
10 NE 10  
10 NE 10

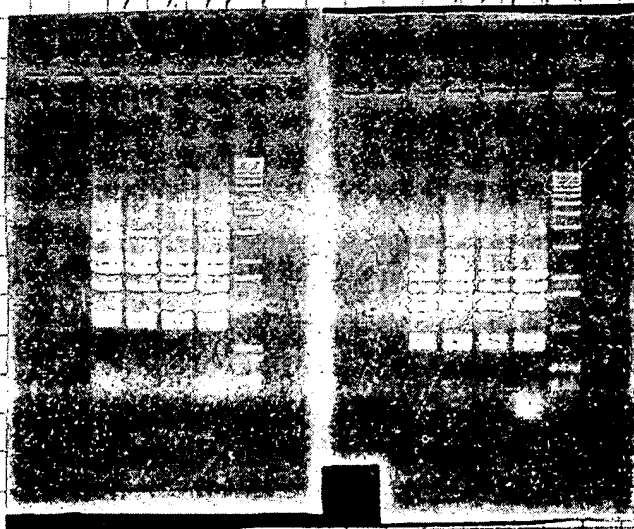
13

NC T-5  
NC T-6

NC TAP

UC TA 3

13 MAY 7  
UC TAY?  
X B 12/100



✓ good - all the clones look as though they contain the correct  $\beta$ AT insert.

It appears that the passage of the CAT activity did not work well (or at all) from the geyser cluster - exposed O/N. Maybe fresh supernatant must be used. Will also try transfection with less RNA / more RNA

**To Page No.**\_\_\_\_\_

Witnessed & Understood by me,

Date \_\_\_\_\_

**Invented by**

**Date**

George W. Kohl

Recorded by,

Recorded by Harold R Clarke

000200

From Page No. 177.Set up  $\phi$ NI ligations at  $12^{\circ}\text{C}$ .

CON.  
 2  $\mu\text{L}$  x 10 buffer  
 2  $\mu\text{L}$  (50 ng) Vector.  
 15  $\mu\text{L}$   $\text{H}_2\text{O}$   
 1  $\mu\text{L}$  T4 ligase

3.8 kb  
 2  $\mu\text{L}$  x 10 buff  
 1  $\mu\text{L}$  of DNA insert ( $\sim 50\text{ ng}$ )  
 5  $\mu\text{L}$   $\text{H}_2\text{O}$   
 1  $\mu\text{L}$  T4 ligase

poured L-Agar - Amp<sup>r</sup> plates ( $100\text{ ng mL}^{-1}$ ) for tomorrow - stored on ice at  $4^{\circ}\text{C}$ Diluted XhoI  $\rightarrow$  Bam. and Bam  $\rightarrow$  XhoI (to  $100\text{ ng } \mu\text{L}^{-1}$ ) and storeddiluted forms at  $-20^{\circ}\text{C}$  stocks at  $-80^{\circ}\text{C}$ .

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne W. Kibbe

Recorded by

David K. Clarke

Nucleocapsid Prips.  
TITLE: Bacterial transformation with 3.8Kb fragment.

Project No. \_\_\_\_\_  
Book No. \_\_\_\_\_

179

From Page No. \_\_\_\_\_

See reference bundle #22A

~~Developed~~ CAT assay entered to see if any CAT activity had been passaged - No luck - will repeat with fresh supernatant next time (probably next week) - pre-infect with helper?

Ran through N-capsid preparation protocol on 10xTC 225s - which were showing good c.p.e. (fusion etc) - lysed in a total vol of 60ml - spun out nuclei - resuspended in 9.5ml total vol of 15% K<sup>+</sup> tartrate - loaded onto preformed 55% - 45% - 35% - 25% gradients in SW 50.1 tubes. Ran at 29K, 5°C for 16 hrs (on at 6:30 pm wed) - off at 10:30 am.

Meanwhile carried out TCID<sub>50</sub> on V<sub>5</sub>H, B9320 undil.

V<sub>6</sub> B9320 dil/undil.

and V<sub>3</sub> 293<sub>1</sub> (A2)

Also did bacterial transformation - T/A cloning kit cells using 5µl of Control and 3.8Kb ligation mixtures for each transformation. Plated out in L-Agar<sup>+</sup> (w/amp<sup>r</sup> Amp).

NB Failure to Phenol extract eluted DNA may prevent efficient ligation!!!

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Steve W. Kline

Recorded by

David R Clarke

000202



George K. Hill

CAT assay of  
passaged (mice)  
supernatant

KODAK SAFETY FILM 340

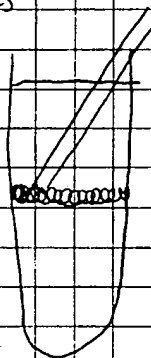
KODAK SAFETY FILM 340

000203

From Page No. \_\_\_\_\_

Checked Potential 3.8Kb clones - it looks as though  
there may be a higher than background level of white colonies -  
indicating the presence of insert DNA!

Also took off N. capsid spin - there were dense flocculent masses lying  
down the centrifuge tubes



Harvested - pelleted in Eppendorf tubes -  
resuspended in NTE - added SDS (0.2%  
final conc - and Proteinase K - 1mg ml<sup>-1</sup>  
final conc.

Let digest at 37°C for 1-2 hrs  
Then phenol chloroform extracted -  
(x2 if necessary)

EtOH ppt<sup>ed</sup> - Noted contamination with  
chromosomal DNA

a look at it Will run an agarose gel tomorrow to take

Set up 12 mini preps (mostly v. small white colonies were selected)  
8 µl of 50 µg/ml Amp / 1 µl l-lysine - Also streaked  
onto a Amp<sup>r</sup> Master plate for growth at 37°C.

Can check potential 3.8Kb clones by BamHI / ~~XhoI~~ ~~Not~~ - neither site  
should exist in the 3.8Kb clone

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Shirley W. Kline

Recorded by

David R. Clarke

Checked Ncapsid Prep-on a Gel.

Project No. \_\_\_\_\_

TITLE: RT/PCR on 4kb Fragment/DMSO/Formamide etc.

Book No. \_\_\_\_\_

181

From Page No. \_\_\_\_\_

Checked growth of Mini preps - many had not grown - will re-do sun → Mon. from well grown master plates.

Spun down 1/2 ml of New N-capsid prep - resuspended lysis pellet in 10 µl x 1 MOPS gel buffer - 10 µl of Denatured Formamide - added 1 µl of 10 mg/ml EtBr and heated at 65°C for 5 mins - proceeded to load gel, including some RNA marker.

Checked RNA gel - there is no clear indication that there is any genomic RNA there - will run a sample through RT-PCR to check if any product is produced.

Set up 4 different RT/PCR RXNS. - Spun down ~ 1.3 ml x 2 for 'old' genomic RNA prep - 1 ml for New prep - resuspended each pellet in 3 µl H<sub>2</sub>O + 3 µl primer - 92°C - 2 mins - 1/2 µl Meth Merc - 5 mins - 1/2 µl Mercaptoethanol - 5 mins → TOOK 4 µl and set up a

- ① Reg RT/PCR with 'old' prep
- ② RT/PCR + 5% DMSO 'old' prep
- ③ RT/PCR + 3% Formamide / 5% Glycerol 'old' prep?
- ④ Reg RT/PCR with New N-capsid prep

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Serge W. Kubi*

Date

Invented by

Date

Recorded by

*David K Clarke*

000205

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE \_\_\_\_\_

Gel analysis of 4kb RT/PCR products.

From Page No. \_\_\_\_\_

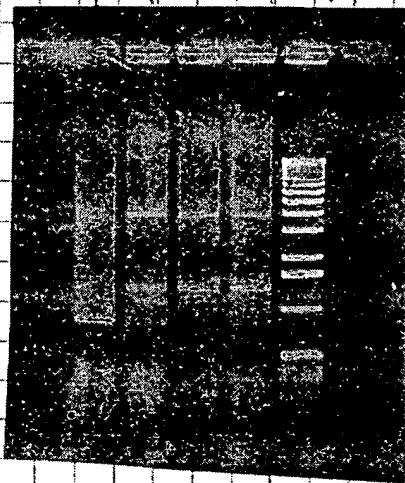
Prepared a 1% agarose gel for analysis of potential 4kb RT/PCR products (Elongation time - now up to 8mins)

Loaded  $\frac{1}{10}$  of each reaction + DNA marker ladder

New Neapid prep  
+ Transmethyl glycerol  
+ DMSO  
reg  
ladder 1kb

Will gel purify the  
4kb fragments tomorrow

Meanwhile - chloroform  
extracted and EtOH ppt<sup>ed</sup>



Good - it looks as though the 4kb RT/PCR product has been produced  $\approx 5-10 \mu\text{g}/\mu\text{L}$ . The New Neapid prep is not working though. The presence of DMSO or Transmethyl glycerol did not appreciably affect yield.

Total yield will be  $\approx 150-300 \mu\text{g}$  in 3 reactions.

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

To Page No. \_\_\_\_\_

Sgt. W. K. Kline

recorded by

David K. Clarke

000206

Gel purification of potential 4Kb DNA fragment  
Set up o/n minis on potential 3.8Kb

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

183

TITLE:

From Page No. \_\_\_\_\_

Set up o/n mini preps on a selection of potential 3.8Kb clones  
(4ml - 100µg/ml Amp = 8µl of stock)  
Will do DNA extractions tomorrow.

Also spun down 4Kb <sup>RT</sup>PCR products - redissolved in 15µl of x1TAElect. Buffer  
and loaded into a 1% Agarose gel, along side marker - in

Proceeded with electrophoresis - Phenol chloroform was extracted after  
elution and ppt'd with 8µl of glycogen Co-ppt't. - There could be  
anywhere from 25-100ng final yield of 4Kb fragment. Will ligate  
total with 2µl (50ng) of vector

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Step W. Kble*

recorded by

*David R. Clark*

000207

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

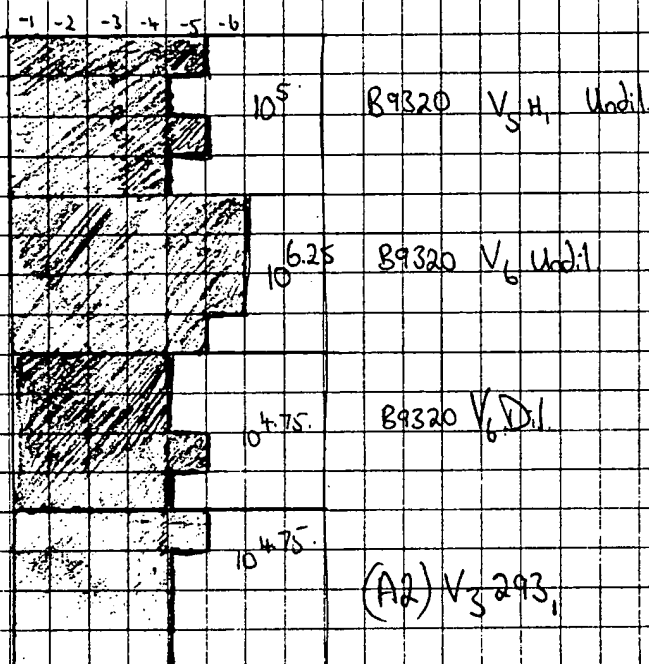
TITLE \_\_\_\_\_

Mini-preps of 5 potential 3.8Kb clones  
Restriction analysis of these 5.

From Page No. \_\_\_\_\_

Checked growth of mini preps - only 5 look as though they have  
grown - will extract DNA later today

Did final read on TCID<sub>50</sub>



Started mini preps - took through to Diet DNA pellet stage - then  
set up BamHI digest in 50µl - 1 1/2 µl enz + 1/2 µl RNase A.

1-2 hrs at 37°C. Then proceeded to analyse on a 1% agarose  
gel

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

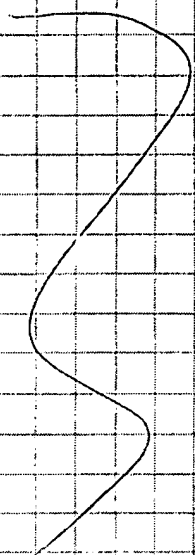
Date

Sgt. W. Kuhl

recorded by

David K Clarke

From Page No. 184.



Good - looks as though N° 4  
may contain the 3.8Kb insert  
(vs. the 3.93Kb) Vector - Proceeded  
with Cla I digestion of the  
Bam HI cut mini prep DNA. -  
Should look for a 659 bp and a  
3141 bp fragment, with the vector  
remaining unchanged.

Set up 0/0 ligation of gel purified 4Kb material with 4Kb Pst II Vector.  
+ Control

2 $\mu$ l x10 buff.  
2 $\mu$ l Vector (500 $\mu$ g)  
15 $\mu$ l H<sub>2</sub>O or DNA 4Kb insert.  
1 $\mu$ l T<sub>4</sub> DNA ligase.  
20 $\mu$ l — 0/0 at 12°C.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

*Steve W. Kuhl*

Date

Invented by

Date

Recorded by

*David K. Clarke*

000209



Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE Cla I cut analysis of potential 3.8kb clone  
Transformation with potential 4kb ligation products

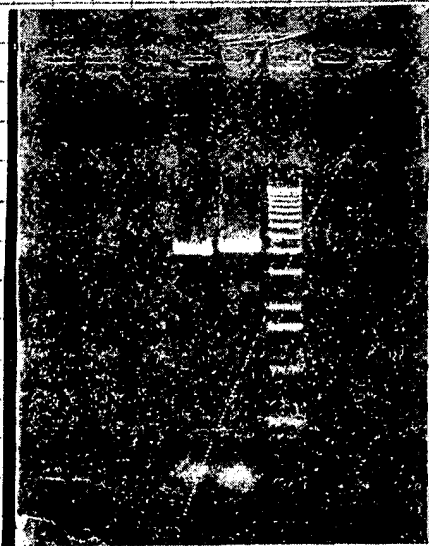
From Page No. \_\_\_\_\_

Spin down Bam HI cut Mini prep DNA - and set up  
 Cla I digestion in topol - 1-2 hrs at 37°C - prepared mini-  
 gel (1%) for analysis.

checked P<sub>2</sub> Vero stocks - have sufficient for another Nicopid prep - will  
 order Hep. 2 cells for Mon.

Ordered 1kb DNA markers from Gibco/BRL

Started transformation with T/A cells - mixed 5µl of each trans ligation  
 mixture with the competent cells and left on ice for ~1hr before heat  
 shocking at +2°C



Cla I digest didn't  
 appear to work,  
 indicating that this the  
 wrong clone, or that the  
 Cla I site may have been  
 lost due to random mutation.

To double check this clone  
 set up 1hr H<sub>ind</sub> III  
 digestions (which has  
 3 cut sites in the 3.8kb  
 fragment)

H<sub>ind</sub> III digests did not indicate any insert either

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

*Steve W. Kibbe*

Recorded by \_\_\_\_\_

*Michael K. Clarke*



TITLE: \_\_\_\_\_

From Page No. 186

Electroporated in 1  $\mu$ l of DNA from Can / 4 kb ligation mixtures

Used 25  $\mu$ l of electrocompetent cells / transformation - used 1.8KV  
at 25 pF capacitance (200  $\Omega$  resistance)

Added  $\approx$  1ml of SOB to each cuvette imm. after electroporation  
and resuspended Bact cells in this - then incubated at 37°C for  
 $\approx$  1hr - plated out 200  $\mu$ l on Amp<sup>r</sup> IPTG<sup>+</sup> X-Gal<sup>+</sup> plates

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sgt. W. Khle*

Recorded by

*David K. Clarke*

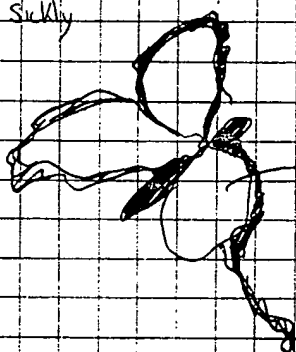
000211

From Page No. \_\_\_\_\_

Checked bacterial transformants - looks pretty good - the electroporation seems to have worked pretty well - there are quite a few colonies on the 'test' plates but not on the CON - ditto T/A.

Put at 4°C to let Blue colour develop, before streaking onto Master plates.

Meanwhile - infected 293 cells with RSV  $V_{H_2}$  32°C ( $10^7$  ml<sup>-1</sup>) - the cells were V. delicate and looked as though they would come off the plate! - Then proceeded with transfection - Under the microscope the cells looked very sickly



long pattern cells all contracted after addition of virus. cells around the edges had come off



remaining cells (v. sick looking)

Meanwhile streaked out 36 potential 4Kb clones from electroporation, and 36 potential 4Kb clones from T/A cloning - + 2 control (five) Bikes

Removed Optimum/transfection/RNA mixture and added 1ml of MEM and left on

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Shy W. Koble

Recorded by

David R. Clarke

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

checked restreaked master plate for potential HK clones - a few clones are not growing well - however there will be plenty to screen - will drop Amp conc to  $50 \mu\text{g ml}^{-1}$  in 2nd cultures.

Meanwhile prepared extracts from transfected 293 cells - as per protocol except prepared final extract in  $50 \mu\text{l}$  Tris (not  $100 \mu\text{l}$ ) since the number of cells was dramatically reduced.

Also did a freeze thaw on one set of transfected cells, to see if that permitted improved transfer of cat activity to fresh 293 cells (which were pre-infected - hr - with  $0.5 \text{ ml}$  of  $V_{2H}$ ,  $32^\circ\text{C}$  @  $10^7 \text{ pfu ml}^{-1}$ ). The preinfecting inoculum was removed prior to addition of transfection supernatant.

Set up 22 mini prep cultures (2 - no controls); 10 from T/A cloning Exp, 10 from Electroporation experiment

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Sgt. W. K. Kile

Date

Invented by

Recorded by

David R. Clarke

Date

000213

From Page No. \_\_\_\_\_

All o/n mini-preps grown - so started mini preps and took through to E-100 p<sup>+</sup> stage; then prepared a 1% agarose gel to do quick ccc analysis along with -ve con and 5Kb insert con.

looked at a total of 18 minis - none contained the 4K insert,

Will screen more minis on Mon - if that doesn't work will do XhoI/Bam digest on PCR product prior to gel purification. Will also do Bam digestion on 3.8Kb fragment prior to gel purification.  
(also no Vector appropriately).

will not do phenol chloroform initially.

Meanwhile prepared cytoplasmic extract from 293 cells which had been infected with 1<sup>st</sup> passage super (freeze thawed super or No freeze-thaw super). Final cell extract contained <sup>was</sup> 100µl. - rapid freeze → -80°C

Also set up reverse transcription on 1µl (span down) of Nucleocapsid prep.

- resuspended in 2µl H<sub>2</sub>O / 2µl primer - heated at ± 80°C 2' → ice, then mistakenly added 2-Mercaptoethanol (mistakenly!!!) - proceeded with rev. transcription anyway ± 1.5hr at 42°C

heat inactivated at 90-95°C for ± 2min  
Then proceeded with PCR rxn

NB Can use KpnI to linearise Vector + Insert

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Steve W. Kuhl*

Date

Invented by

Date

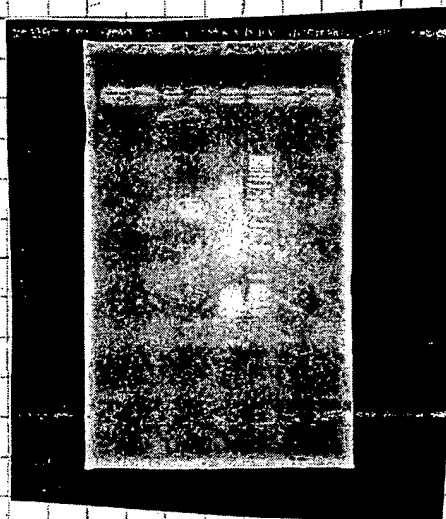
Recorded by

*David K. Clarke*

From Page No. \_\_\_\_\_

Prepared 1% Agarose gel for analysis of RT/PCR products  
(Hopefully 3.8Kb fragment) Loaded 10 $\mu$ l (of rxn) for analysis

Will have not more than  
25ng of 3.8Kb band  
after gel purification - will  
ligate with 25ng of Vector  
in 10 $\mu$ l



Surprisingly there is a 3.8Kb  
PCR product! Phenol  
chloroform extracted and  
EtOH ppt<sup>ed</sup> with 3 $\mu$ l of  
glycogen as Co-ppt<sup>nt</sup>

Will try to gel purify

The Bam HI digestion should improve the cloning efficiency  
of the 3.8Kb frag.

tomorrow after Bam HI  
digestion (will also Bam digest  
same Vector)

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sege W. Kibbe*

Recorded by

*David K. Clarke*

From Page No. \_\_\_\_\_

Spin down 3.8kb RT/PCR product - dried - and set up Bam HI  
digestion in 20 $\mu$ l; along with PCR II Vector - gave 1.5hrs at  
37°C (used 1 $\mu$ l Bam for each digestion)

Meanwhile prepared a 1/5 agarose gel for electrophoresis - loaded and run  
for  $\approx$  45mins with marker DNA; - Proceeded to electrophore DNA onto

NA-45 paper - 6mins - a little too short for the PCR II Vector - only  
got  $\approx$  1/2 (100ng) - Eluted into 120 $\mu$ l total of high salt NET -

Diluted x2 and Phenol Chloroform extracted - then ppt'd with 10 $\mu$ l of  
glycogen as Co-ppt<sup>nt</sup>. Would estimate max recovery at 25-50ng for  
3.8kb band and 100ng for Vector - will set up 1:1 ratio for ligation.  
(all of 3.8kb material  $\approx$  25ng with 1/4 of Vector in 15 $\mu$ l ligation rxn)

Meanwhile set up another 2 $\times$  minipreps, with potential 4K clones - Amp<sup>r</sup>  
- will run minis tomorrow

Need a new book!

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Sgt. W. K. Kline*

Date

Invented by

Date

Recorded by

*David K. Clarke*

# TITLE

Mini prep analysis of Potential 4Kb clones.  
CAT assays of P<sub>0</sub> and P<sub>1</sub> RSV/CAT transfections - A helper.

Carried out another 24 mini preps on potential 4K clones - prepared 2x 1 1/2 Agarose gels, each with 2 combs. Omitted phenol chloroform extraction to save time. Ran 1/5 of each prep - no luck - there were no 4K-insert-sized plasmids.

Meen time infected VeroS (new) with 'V.P. B9320 und.1' - did an undil + 10<sup>-1</sup> dil infection (0.25ml on 25cm<sup>2</sup> Flasks) - adsorbed ~ 3hrs - then fed with 4ml of MEM.

Also carried out CAT assays using the four previously prepared cell extracts

① 1/2 RNA (reduced amount of transfecting RNA.

② No freeze Thaw.

③ No freeze Thaw passage ①.

④ ~~Freeze~~ Freeze-Thaw passage ①.

Used 20µl of Extract for each reaction - let run for 2-3hrs at 37°C - freeze away at -80°C prior to ethyl acetate extraction and TLC.

Witnessed by: Aye W. Khle.

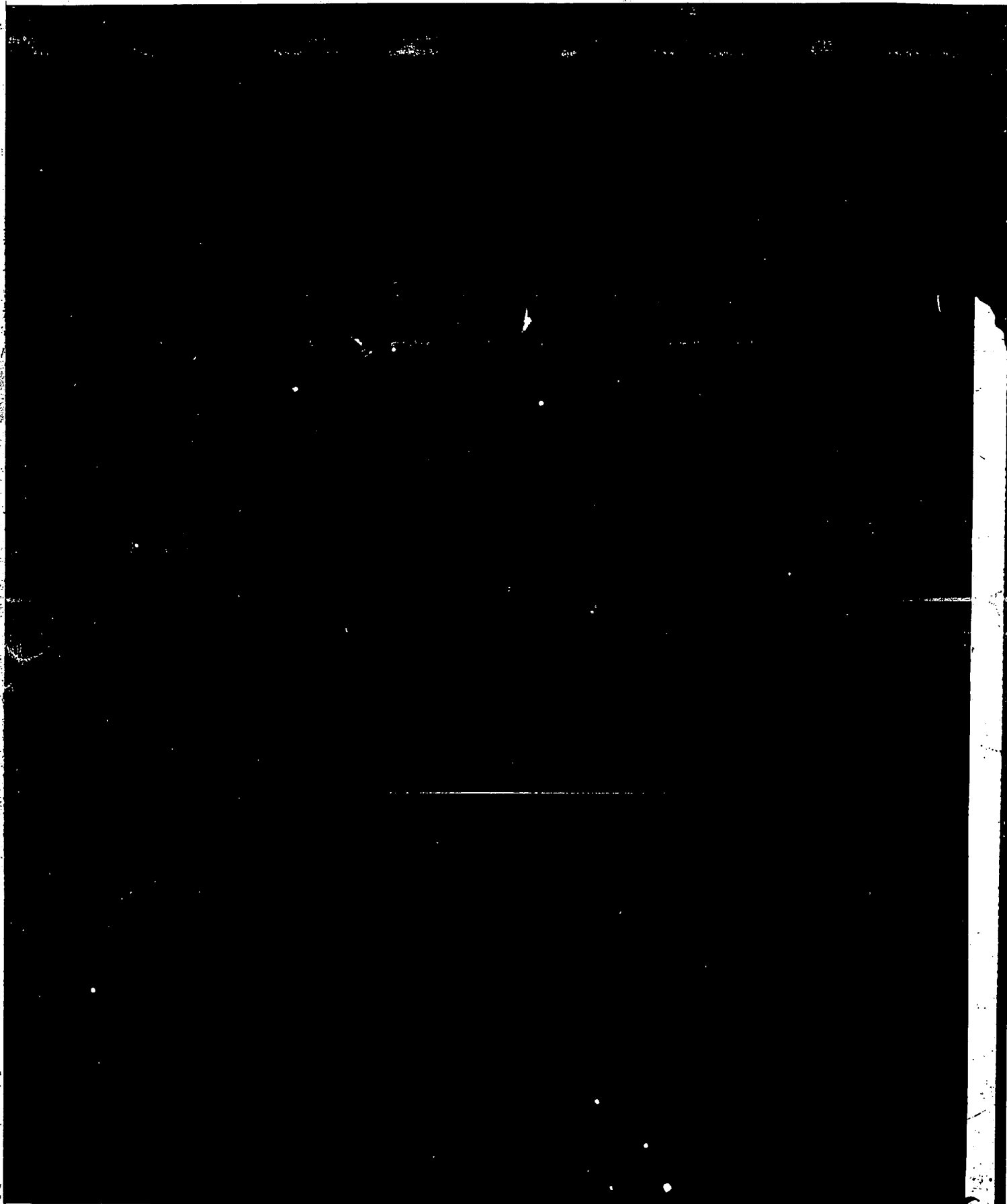
Recorded by: David K. Clarke

DATE

000217

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000219

BOOK 2

LABORATORY  
BOOK

DAVID CLARKE

ANALYST

0000220

Associated Binder #045A

000221

NOTEBOOK NO. #045  
ISSUED TO DAVID CLARKE  
ON \_\_\_\_\_  
DEPARTMENT \_\_\_\_\_  
RETURNED \_\_\_\_\_

—SCIENTIFIC NOTEBOOK CO.—  
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P.O. BOX 238  
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000222

## INSTRUCTIONS

1. The primary purpose of this notebook is to protect your and the Company's Patent-Rights by keeping records of all original work in a form acceptable as evidence if any legal conflict arises.
2.
  - When starting a page, enter the title, project number, and book number.
  - Use ink for permanence -- avoid pencil.
  - Record your work as you progress, including any spur-of-the-moment ideas which may be developed later.
  - Avoid making notes on loose paper to be recopied.
  - Record your work in such a manner that a co-worker can continue from where you stop. You might be ill and to protect your priority it could be urgent that the work continue while you are absent.
3.
  - Give a complete account of your experiments and the results, both positive and negative, including your observations.
  - Record all diagrams, layouts, plans, procedures, new ideas, or anything pertinent to your work including the details of any discussions with suppliers, or other people outside the Company.
  - Do not try to erase any incorrect entries; draw lines deleting them, note the corrections, sign and date the changes. This extra care is worthwhile because of the necessity of original data to prove priority of new discoveries.
4.
  - After entering your data, sign and date the entries.
  - Explain your work to at least two witnesses who are not co-inventors, and have them sign and date the pages in the place provided.
- Record the names of operators and witnesses present during any demonstration and have at least two witnesses sign the page. If no witnesses are present during an experiment of importance, repeat it in the presence of two witnesses.
5. Since computer programs can be patented these instructions apply to the development of computer software. In this case a description of the structure and operation of the program should be recorded in the notebook, together with a basic flow diagram which illustrates the essential features of the program. In the course of developing the code, the number of lines of code written each day should be recorded in the notebook, together with a statement of the portion of the flow diagram to which the section of code is directed.
6. This notebook and its contents are the exclusive property of the Company. It is confidential and the contents are not to be disclosed to anyone unless authorized by the Company. You must return it when completed, upon request, or upon termination of employment. It should be kept in a protected place. **If loss occurs, notify your supervisor immediately, and make a written report describing the circumstances of the loss.**





































# TLC analysis of CAT Transfections.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

EtHyl Acetate extracted + CAT assays from yesterday;  
dried down in the speed vac - meanwhile equilibrated TLC Tank  
with solvent.

Checked CPE on 293 cells - nothing much yet - will check again  
this afternoon

Loaded TLC

LHS → RHS

1 RNA  
2

NO RT

RT  
RT Passage

NO RT  
RT Passage

Took off after ~ 2 hrs - and put away for autoradiogram.

From the sound of the Geiger counter, there should be significant CAT activity.

Meanwhile prepared X6 Amp<sup>r</sup> agar plates 100 µg/ml<sup>r</sup>  
X3 Kan<sup>r</sup> agar plates 50 µg/ml<sup>r</sup>  
passed - dried and stored at 4°C until tomorrow.

Meanwhile set up o/n ligations with Bam HI cut 3.8K PCR product  
which had been gel purified. used all in single ligation

CON

2 µl Vector (25 µg)

1 µl X10 LMP

6 µl H<sub>2</sub>O

1 µl T4 ligase

20 µl

3.8K

2 µl Vector

1 µl X10

6 µl insert

1 µl T4 ligase

20 µl

O/N 12°C

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Sup W. Kibbe

Recorded by

David K Clarke

000240

From Page No. \_\_\_\_\_

Checked c.p.e. on 89320 infected Vero cells - progressing nicely - will harvest later today.

Developed RV-CAT Transfection protocol - looks good

① There was a v. high CAT activity following the initial transfection - event when the transfecting RNA was cut in half.

② CAT activity can be assayed either directly from supernatant from transfected cells - without any processing, OR following freeze-thaw of transfected cells.

Set up transformations - Spl of each ligation (con + 3.8K) was used to transform T/A cells

Spl of each ligation was used to transform Electro cells

Meanwhile harvested 89320 D./undil. c.p.e. was extensive in both, but was a little more pronounced in undil.

Freeze down at  $-80^{\circ}\text{C}$ .

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sign W. Kelle*

Recorded by

*David K Clarke*

000241

TITLE: Planning future work (for Cloning Viral stocks etc.)

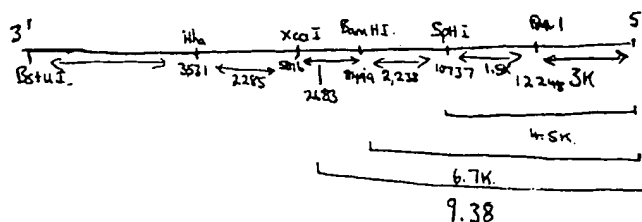
Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

3

From Page No. \_\_\_\_\_

Checked for bacterial transformants - No  $\phi$  - only one transformant was present on an Amp plate - will check it out by mini prep tomorrow.



Revised cloning procedure for genome of RSV.

prepared Amp<sup>r</sup> plate to restreak clone banks.

checked RSV A2 ATCC pV, p<sub>2</sub>V etc. - will order:

1 x 75cm<sup>2</sup> Vero for P<sub>1</sub> → P<sub>2</sub> Vero (ATCC)  
 2 x 75cm<sup>2</sup> Vero for P<sub>2</sub> → P<sub>3</sub> Vero (ATCC)  
 2 x 25cm<sup>2</sup> Vero <sup>DS</sup> for P<sub>1</sub> → P<sub>2</sub> Vero / P<sub>2</sub> → P<sub>3</sub>.

Designed SphI → PstI cDNA primer

5'-ACA/TAT/AGG/CAT/GCA/CCG-3'

17/18-Mer

502 m.p.

Plated out clone banks and grow O.N. at 37°C.

19  
32  
50

Also set up single mini prep of potential 3.8K clone.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sign W. Kille*

Recorded by

*David K Clarke*

0000242



Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Mini prep analysis of potential 3.8K clone.  
T.C.I.D.<sub>50</sub> for B9320: Attempted Plaque Assay

From Page No. \_\_\_\_\_

Came out mini prep DNA extraction of potential 3.8K clone - ran a sample on a 1% Agarose gel alongside a 3K control. No-luck - it did not contain a 3.8K insert - will resort to cloning smaller fragments.

Meanwhile did T.C.I.D.<sub>50</sub>s on B9320 - V<sub>7</sub> Dil/Unit.

Also infected 25cm<sup>2</sup> Vero monolayers with 10<sup>-2</sup>, 10<sup>-3</sup> and 10<sup>-4</sup> Diluted P<sub>3</sub>V virus for plaque assay (used 200µl of each) - Adsorbed 2-3hrs and then overlaid each with 6-7ml of 0.4% Agar/EMEM. Allowed to solidify at Room Temp for 20mins, then placed at 37°C. Ordered SpH<sub>1</sub> > PmLI Oligos.

Frank's Meeting at 4pm.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Lutz W. Kibbe

Recorded by

David K. Clarke

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

5

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Prepared Alex's letter of recommendation for Graduate school.

Stored bacterial clone banks at 4°C.

Checked *At. boliviae* in vivo plaque assays. - seems fine.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Greg W. Kohn*

Recorded by

*David K. Clarke*

000244

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE Infection of Hep-2s for Nucleocapsid prep.

From Page No. \_\_\_\_\_

Ordered 3 x 96 well plates for fin. to fibrate Vero stocks.

Infected 10 x TC 150s with 1ml each of either  $P_{3V}$  or " $P_{3H}$ , 32°C"

adsorbed  $\approx$  3hrs at 37°C 10am - 1pm. then put at 32°C for 4hrs

prepared NA 45 paper for electrophoresis of DNA bands - stored at 4°C

Spent the afternoon preparing 2-monthly report.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kelle*

Recorded by

*David K. Clarke*

TITLE: General virology observations.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

7

From Page No. \_\_\_\_\_

Checked TCID<sub>50</sub> for V<sub>7</sub> B9320 Undil/Dil (4 days p.i.)

It is clear that Undil is still better than dil for infection. It looks as though the titer is going to be  $\approx 10^{5.5}$  for Undil (lower for dil) will make a final reading on Thurs. Should try infecting Vero at 32°C and/or infecting Vero DC-5 at 37°C/32°C all with P<sub>6</sub> or P<sub>7</sub> V B9320.

Checked plaque assay of A2 B3V - small plaques are now visible with the naked eye - will leave until Thurs to get a final count.

Checked c.p.e. progression on Hep-2 cells at 24 h.p.i. - There are some signs of c.p.e. on the Hep 2s. will check again this afternoon and hopefully they will be ready to harvest by tomorrow - late morning.

Prepared some new 55% w/v K<sup>+</sup> Tartrate.

Will run under

✓ SphI to BamHI 5'-GGT/GCA/TGC/CTA/TAT/GT 17mer 50°C m.p.  
 ✓ XcaI to BamHI 5'-TGG/~~TTG~~/TTG/GTA/TAC/CAG/TGT/T 19mer 54°C m.p.  
 ✓ HhaI to XcaI 5'-TTT/ACC/ATA/TGC/GCT/AAT/GT 20mer 54°C m.p.  
 ✓ XcaI to HhaI 5'-AAC/ACT/GGT/ATA/CCA/ACC/A 19mer 54°C m.p.

To Page No. 8

Witnessed & Understood by me,

Date

Invented by

Date

*Serge W. Koble*

Recorded by

*David K. Clarke*

0000246

From Page No. 1

✓ BstUI to HhaI

GT

5'-ACG/CGA/AAA/AAT/GCG/TAC/A

19mer. 5'c mp

✓ HhaI to BstUI

Should not  
migrate  
negatively.

5'-ACA/TTA/GCG/CAT/ATG/GTA/AA

20mer 5'c mp

Prepared: K<sup>+</sup> tartrate solution + lysis buffer for nucleoside prep

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sign W. K. K. K.

Recorded by

David K. Clarke

TITLE: Nucleocapsid Preparation

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

9

From Page No. \_\_\_\_\_

Proceeded with nucleocapsid prep - (did lysis step in 10ml/5 TC-150)

Infected 3x 75cm<sup>2</sup> Vero's → Not very confluent!

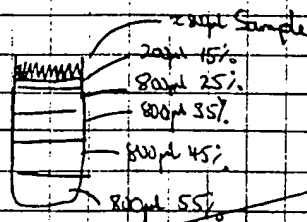
Two with P<sub>2</sub> V ATCC } 0.25ml inoculum each.  
One with P<sub>1</sub> V ATCC

Also infected 2 x Vero-DC-5

Both with V<sub>6</sub> B4320 unadl → 37°C  
→ 32°C.

Screened up Nucleocapsid prep - forgot to spin out nuclei

However - tried to compensate by addition of RNase free DNase (to digest nuclear DNA - mixed in 10µl per tube prior to T.E spin) - Resuspended each pellet in 2ml 10mM Tris, 1mM MgCl<sub>2</sub> - added another 5µl of DNase and incubated on ice for 2-3 hrs - after 1 hr added 500µl of 55% K<sub>2</sub>tartrate. Meanwhile prepared gradients



Witnessed & Understood by me,

Date

Invented by

Date

To Page No. \_\_\_\_\_

*Shirley W. Koble*

Recorded by

*David K. Clarke*

000248

From Page No. \_\_\_\_\_

Checked plaque assay 6 days post infection - plaques are well developed stained with Crystal Violet after picking out the agar. The plaques are uneven and should probably have been stained at day 5 p.i. (possibly day 4) - also should perhaps use 5ml (instead of 5ml) per well MEM to decrease slippiness slightly. The titer was  $\approx 3 \times 10^5$  pfu/ml<sup>-1</sup> calculated from  $10^{-4}$  dilution. This value was slightly higher than that obtained for the TCID<sub>50</sub> (as expected).

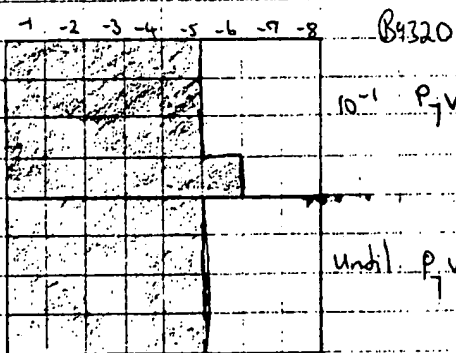
Took off nucleocapsid spin - Presence of genomic RNA does seem to be a problem - harvested the white, flocculent minor band close to the major band of material - spun down in eppys - SDS-Prot K treated pellet - phenol chloroform extracted and etOH ppt'd.

Meanwhile spin down RNA (genome) preps for cDNA/PCR rxns.

Did final read on TCID<sub>50</sub> B9320 P<sub>7</sub>V - it appears that infection with



P<sub>6</sub>Vero 10<sup>-1</sup> gave a slightly higher titer vs P<sub>6</sub>Vero Undel.



Also checked DC-5 infected Vero's 32°C/37°C - both are showing c.p.e.

as are the Vero's infected with ATCC P<sub>1</sub> and P<sub>2</sub> Vero

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sege W. Kibbe*

Recorded by

*David K. Clarke*

TITLE: \_\_\_\_\_

From Page No. 10

Set up RT/PCR runs on three different samples - using SphI  $\rightarrow$  PstI as primer (m.p. 50°C + 17mer) (10 1.5M Mg)

Reduced 72°C phase to 5mins (for the shorter fragment)

Did another check on c.p.e.

Very noticeable that 37°C B9320 until P<sub>6</sub>V infection has much more pronounced c.p.e. than 32°C infection.

Witnessed &amp; Understood by me,

Date

Invented by

Date

To Page No. \_\_\_\_\_

*Sgt. W. K. K. K.*

Recorded by

*David K. Clarke*



Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE Analysis of 1.5K RT/PCR Rxn

From Page No. \_\_\_\_\_

Checked c.p.e. on infected Vero's - all looks good - will harvest

later this afternoon. c.p.e. looks v. good on DC-5 at 37°C

took off RT/PCR Rxn. Loaded 10 µl samples each of

(L.H.S.) Lad/Old/NEW/?/



The lower PCR band is the one that I want - will probably purify both and select later, or can set up electroelution to enrich for lower band i.e. run out as far as possible, then elute for a short period of time (3-5 mins).

Chloroform extracted and EtOH pptd with 5 µl of stock glycogen as 100 pp<sup>nt</sup> - will gel purify on sun.

Would estimate 20-50 ng in 1.5K band. Should give 10-25 ng following purification.

Proceeded with virus harvests and TCID<sub>50</sub>s.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kibb*

Recorded by

*David K. Clarke*

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Spun down RT/PCR for 1.5K fragment - Prepared 1% Agarose gel for purification of DNA.

Ran out - long run - stained - eluted DNA onto NA45 paper then eluted off in high salt - Phenol/chloroform extracted and ppt'd with glycogen

Decided to harvest both DNA bands onto NA45 paper (1 piece) - just in case! Also will add bulk - minimising losses.

Trimming out cloned DNA with EcoRI should give 3 fragments. 3.8K Vector  
600 b.p frag  
924 b.p frag

Ordered 4 Oligos indicated on page ⑦

Witnessed &amp; Understood by me,

Date

Invented by

Date

To Page No. \_\_\_\_\_

*Steve W. Kille*

Recorded by

*David K. Clarke*

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

Infection of Heps for Nucleocapsid.

TITLE On ligation of 1.5K Frag.

From Page No. \_\_\_\_\_

⊗ Ordered T/A cloning kit. ⊗

Infected 10 x TC150s each with  $\approx$  1ul of either  $V_3$  E or  $V_3$  H<sub>1</sub> B1cc  
adsorbed 3hrs at 37°C - then put at 32°C.

Meanwhile set up own ligationResuspended purified 1.5K material in 8ul H<sub>2</sub>O

Set up R.N.S.

2ul Vector (50ng) 1:1 ratio  
2ul Insert ( $\approx$  25ng)  
2ul x10 buffer  
13/15ul H<sub>2</sub>O (rest/conc)  
1ul T<sub>4</sub> DNA ligase  
20ul  $\rightarrow$  on 12°C.

prepared 9 Amp<sup>r</sup> plates for transformation. - Poured - stored at 4°C

Prepared x-gal (4mg/ml) in Dimethyl formamide - stored at -20°C in  
silv silver foil wrapping

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne W. Koble

Recorded by

David K. Clarke

From Page No. \_\_\_\_\_

Checked c.p.e. on infected Hep-2s - Those infected with  $V_{3H_1}$  are showing a higher level of dead cells than those infected with  $P_3V$  where there is not much indication of viral c.p.e. yet. Will check again later this afternoon.

Prepared fresh lysis buffer for nucleocapsid prep.

Preliminary check on TCID<sub>50</sub>s indicates

- ① DC 5 Vero's are not significantly better for growing B9320
- ② 32°C may be better than 37°C for growing B9320 (should be tried on 'regular' Vero's)

Titre

Will order some Hep-2 cells for  $V_{3H_1}$  stocks. 3 x 75cm<sup>2</sup>

Also some regular Vero's for B9320 32°C growth cf. 37°C growth  
Dil/well at each temp. 4 x 25cm<sup>2</sup>

Carried out transformation of 'one-shot' bacteria from Tarragon  
Used 5 µl from each gyl ligation (plastic residue from Dantrolene/formaldehyde dissolution of plastic container used to make up X-gal may inhibit bacterial growth)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kelle*

Recorded by

*David K. Clarke*

From Page No. \_\_\_\_\_

Virus infected Hep-2's were showing a lot of c.p.e. - initiated

Nucleocapsid production - including Nuclei/Nucleus spin

Checked bacterial transformants - there are some whites on the test plate - less on the control - will streak out on a master plate <sup>QAS</sup>.

Ran through nucleocapsid prep - resuspended <sup>each</sup> crude N. capsid pellet in 2.4ml.

15% (w/v) K<sup>+</sup> tartrate - Meanwhile prepared K<sup>+</sup> tartrate gradients

Did lysis steps with <sup>10ml</sup> ~~5ml~~/pellet 5 TC 150s.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sgt. W. K. Kline*

Recorded by

*David K. Clarke*

TITLE: Nucleocapsid harvest / O/N Minis on Pt. 1.5K clones

Project No. \_\_\_\_\_  
Book No. \_\_\_\_\_

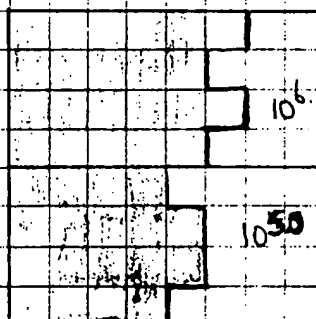
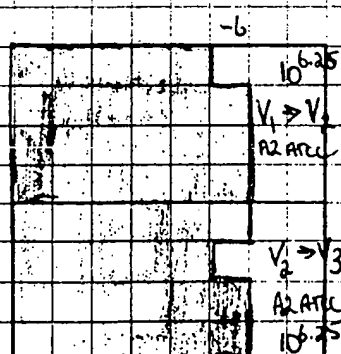
17

From Page No. \_\_\_\_\_

Checked transfections (streaked on master plate) About  $\frac{1}{2}$  are showing some signs of blue colour - indicating absence of an insert - will pick a few bluish ones + the remainder of white ones and set up O/N mini preps - Did 14 minis (4 or 5 look as if they may be true)!

Meanwhile took off nucleocapsid spin and harvested bands. Resuspended 5 Plaks worth in 1ml final vol. I.E. buffer and carried out SDS/proteinase K digestion for 1 hr at 37°C followed by phenol. Chloroform extraction and ethanol ppt.

Final TC2050 Reads



B9320  
V<sub>6</sub> DC5, 32°C

B9320  
V<sub>6</sub> DC5, 37°C

Will use in Rescue Attempt

Better growth temp. at least in DC5s.

These titres are good and consistent with previous titres at the same passage.

Diluted out and stored new Oligos

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sgt. W. K. K. K.*

Recorded by

*David R. Clarke*

0000256

From Page No. \_\_\_\_\_

Carried out mini prep analysis of 14 possible 1.5K clones - ⑦ and ⑩ had not grown so well, so can expect to get DNA from only 12 (of those 4 had blue colour tint - so narrow field down to 8 possibles)

Meanwhile prepared a 1% gel for analysis of mini preps

Ran the gel while in meeting with Laura Coruzzi - unfortunately it ran too far - Proceeded to Phenol Chloroform extract the mini preps and then etch plates

Spun down new N-capsid prep RNA - it looked good - dried and stored at -20°C in preparation for reverse transcription/PCR tomorrow

Meanwhile spun down plasmid DNA from mini-preps and set up EcoRI restriction digests in total final vol. ~1µl RNase A and 1µl EcoRI ~1hr at 37°C.

loaded half of each digest on a 1% Agarose gel with a 1Kb ladder - checked gels - No luck - there does not appear to be any inserts at all.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne K. K.

Recorded by

David K. Clarke

TITLE

RT/PCR for BamHI/SphI fragment.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

19

From Page No. \_\_\_\_\_

Set up RT/PCR reactions on RNA from resect nucleocapsid preps  
(V<sub>3</sub>, and V<sub>3</sub>H, derived nuc.)

Resuspended each pellet in 3  $\mu$ l H<sub>2</sub>O and 3  $\mu$ l Bam  $\rightarrow$  Pml primer - 90°C for  
1.5 mins  $\rightarrow$  Ice  $\rightarrow$  1/2  $\mu$ l of Math. Nuc  $\rightarrow$  RT for 3 mins  $\rightarrow$  1/2  $\mu$ l of  $\beta$ -mercap.  $\rightarrow$

RT for 3 mins  $\rightarrow$  Rxn mixture for 1-2 hrs with Rev. Trans, 42°C

Heat inactivated at 95°C for 2 mins - then proceeded to set up PCR

Rxns using ~ 0.5-1.0  $\mu$ l of T<sub>aq</sub>

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sgt. W. K. K. K.

Recorded by

David K. Clarke.

000258



From Page No. \_\_\_\_\_

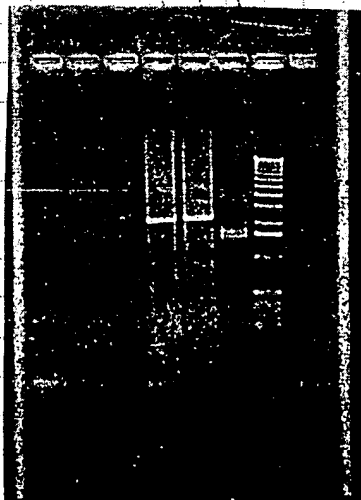
Set up o/n ligation of gel purified 1.5Kb frag. and PCR II vector

2 $\mu$ l of Vector4 $\mu$ l Inert (saved 2 $\mu$ l for gel analysis)2 $\mu$ l X10 buffer11 $\mu$ l H<sub>2</sub>O1 $\mu$ l T<sub>4</sub> DNA ligase20 $\mu$ l

put at 12°C o/n

analysed 10 $\mu$ l from each O/N RT/PCR RAN also 2 $\mu$ l of purified 1.5Kb  
alongside the 1Kb ladder. (looking for a 2.25Kb fragment)

5 $\mu$ l } 2.25Kb RT/PCR  
 10 $\mu$ l }  
 1.5Kb gel purified DNA  
 1Kb ladder

Chloroform extracted  
RT/PCR products andEtOH ppt<sup>ed</sup> along with

V good - looks as though

both nucleosidic preps are  
 yielding good genomic template  
 and there is abundant 2.25Kb  
 RT/PCR product. Bam/Sph...

Will proceed with other RT/PCR  
 RANs as soon as possible - next  
 week sometime (possibly Wed)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suz W. Kibbe

Recorded by

David K. Clarke

TITLE: Transformation of bacteria with 2.25Kb frag. 1.5Kb

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

21

From Page No. \_\_\_\_\_

Prepared 30ml L-Agar - autoclaved

Infected Hep-2 cells with P3V (each 75cm<sup>2</sup> flask with 0.75ml virus)

Also infected Vero with V<sub>6</sub> DC5-1 (each 25cm<sup>2</sup> flask with 0.25ml)

adsorbed  $\approx$  3hrs at 37°C

Prepared a 1% agarose gel for purification of 2.25Kb fragment.

Carried out bacterial transformation with ligation mixture containing 1.5Kb fragment. Left on ice for 1hr then heat shocked at 42°C for 30secs prior to incubation in SOC for phenotypic expression (1hr 37°C).

Spin down 2.25Kb PCR products - combined both V<sub>3</sub> and V<sub>3</sub>HT, DNA and

run out on a 1% Agarose gel - purified onto NA45 paper and then eluted

- pptd with 3 $\mu$ l of glycogen. - There appeared to be plenty of DNA in the

Prepd  
clonform  
extracted 2.25Kb band (should have  $\approx$  1 $\mu$ g)  
after purificat.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Sgt. A. R. R. R.

Recorded by

David R. Clarke

0000260

From Page No. \_\_\_\_\_

Checked bacterial transformants - looks good; these are the 'right' number.

Checked 293 cells - they are not ready - will try setting up some myself O/N for use tomorrow.

Checked c.p.e. on Vero cells infected with B5320 - beginnings of c.p.e. apparent (more so at 37°C as expected and more at undil. v. dil). Will check again this afternoon.

Hep-2 infected with V<sub>3</sub> - some c.p.e. apparent - cells were initially only 60-70% confluent and may not yield as much virus - will check again this afternoon.

Set up some fresh 293 cells - Took a TC 150 and trypsinized into 2ml of

Tryp-EDTA. Added 1ml to 18ml medium and plated out into 6 wells of a 6 well plate (3ml/well). Similarly added 0.5ml cell sus. to 6ml of MEM and plated into 2 wells, and finally added 0.1ml cell suspension to 6ml MEM and plated out (1/2 @ shake plates) 37°C O/N.

Initiated RT/PCR rxns for Xca to Bam (2.68K bp) and Hha to Xca (2.28K bp).

Set up Rev transcriptions as before - let rxn for  $\approx 1.5$  hrs.

Then proceeded to heat inactivate and set up PCR.

Meanwhile set up O/N mini preps x10 in Amp<sup>r</sup> L-B

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne W. Kille

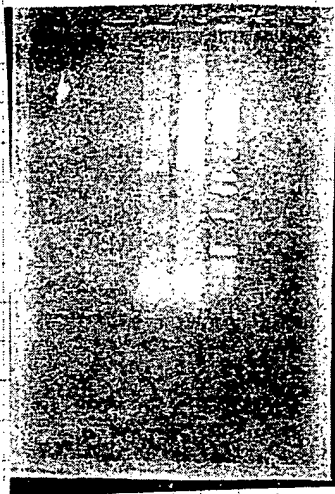
Recorded by

David K. Clarke.

0000261

From Page No. \_\_\_\_\_

Run 1% Agarose gel to analyse RT/PCR products for Xcu/Bam and Hha/Xca



V. good - both products are as expected and in fairly good abundance.

500ng lesser band (total prod)  
1µg greater band (total prod)

Chloroform extracted and EtOH ppt<sup>d</sup> with 1µl glycerol - Can gel purify tomorrow.

Carried out Mini preps in search of potential 1.5K clone (sph/Pml)  
~~(Still need to do Bam/Sph synthesis - will do when doing 3' fragments)~~

Did EcoRI digestion to check 1.5K insert. - ran 2 mini gels to check for insert.

Carried out transfection of B9320 (V<sub>6</sub> DE5, 32°C) infected (1ml ≈ 1.5hrs) 293 cells (90% cell culture)

Used ≈ 12µl of In Vitro transcribed RNA (some from earlier IVT ≈ 6µl) for transfection.  
left transfection mixture on for 4-5hrs at 37°C - then replaced with 1ml MEM - left at 37°C ON.

Meanwhile harvested V<sub>3</sub> H<sub>1</sub> 32°C ATCC A2 virus for working stock. Also harvested B9320 DI/undl at 32°/37°.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kuhl*

Recorded by

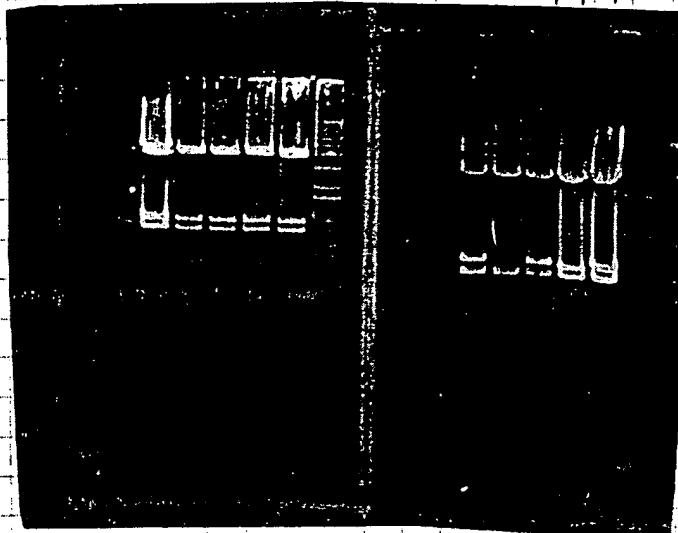
*David R Clarke*

0000262

From Page No. 23

Continued from other page

3/21



Good gel shows  
both 1.5 and 1.6K  
inserts with central  
EcoRI as expected  
however will re-cut clones  
①, ② and ③ to check  
insert size more carefully.

It looks as though ② and  
③ represent both predicted  
bands.

Size difference in 924 bp fragment between  
1.5K and 1.6K clones indicates that the size  
difference is generated at the SphI priming site.

Will cut with BamHI on  
one side and NotI or XhoI on the other  
but will keep entire clone bank for the moment.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Steve W. Kuhl

Recorded by

David K. Clarke

0000263

TITLE:

Gel purification of RT/PCR Products Xca/Bam

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

25

From Page No. \_\_\_\_\_

Prepared 1% Agarose gel for purification of Xca/Bam and Hha/Xca RT/PCR products.

Checked infected/transfected 293 cells - they look pretty sickly and there is evidence of c.p.e. - will harvest cell extracts

Ordered BstNI to Hha I and Hha I to BstNI

Proceeded to elute RT/PCR products from the gel and then phenol chloroform extracted and etOH ppt'd with glycogen

Will analyse all three purified DNAs (1/2 of each prep) on a 1% agarose gel over the weekend

Extracted cytoplasmic contents from transfected 293 cells - froze in EtOH/Dry ice and stored at  $-80^{\circ}\text{C}$

Will assay on Sat or Sun

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Krole*

Recorded by

*David K. Clarke*

0000264

From Page No. \_\_\_\_\_

Give RSV presentation to S.A.B. 10-45 am

Carried out Bam HI digestion on 1, 2, 3, 8 (mini prep DNA samples)  
to further characterise DNA insert size - gave ~1.5 hrs at 37°C then  
phenol chloroform extracted and EtOH ppt<sup>ed</sup>.

Meanwhile carried out TCID<sub>50</sub>s on latest B9320 populations

1) a) V<sub>6</sub>DC5, V<sub>1</sub>, 37°C Undil.

b) V<sub>6</sub>DC5, V<sub>1</sub>, 32°C Dil.

2) a) V<sub>6</sub>DC5, V<sub>1</sub>, 32°C Undil.

b) V<sub>6</sub>DC5, V<sub>1</sub>, 32°C Dil.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Koble*

Recorded by

*David K Clarke*

TITLE: CAT assay of 'B' Rescue

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

27

From Page No. \_\_\_\_\_

Set up XhoI digest on 1.5K mini prep DNAs (1, 2, 3, 8)  
which had been previously cut with Bam HI - Cut for ~1.5hrs and then  
freeze away prior to gel analysis tomorrow

Also set up CAT assay using 20µl of 'B' extract - let  
run for ~3hrs, then freeze at -80°C prior to TLC analysis  
on Mon.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sgt. W. Kille*

Recorded by

*David R. Clarke*

000266



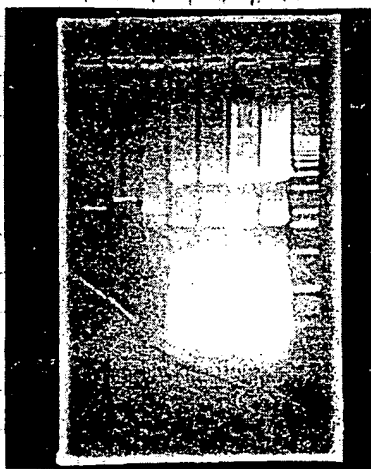
Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

Quantitation of Purified RT/PCR products  
TITLE Gel analysis of Bam/Xho cut Minis (1.5K)

From Page No. \_\_\_\_\_

prepared 1% agarose gel to analyse Bam/Xho cut mini prep DNAs (for identification of correct cDNA clone) - also ran 1/3 of each gel purified RT/PCR product off from further along the genome, to quantitate prior to setting up ligations.



\* No 2 is the correct clone for the 1.5K insert. - will look for others at a later date as back-up.

2.25K band contains  $\approx$  200ng of DNA  $\approx$  30ng  $\mu$ l<sup>-1</sup> - will use 2  $\mu$ l for ligation.

Xba to Bam band contains  $\approx$  10ng. Will need to use  $\approx$  10-12  $\mu$ l for ligation. - ditto Hlu to Xba.

proceeded to set up ligations with appropriate amounts of Vector + insert

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

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Date

Signed W. K. Clarke

Recorded by

David K. Clarke

From Page No. \_\_\_\_\_

Ethy Acetate extracted CAT assay material and dried down in the Speed Vac.

Equilibrated the TLC tank ~2hrs prior to running the TLC.

Meanwhile prepared 100ml L-Agar for transformations - poured plates - dried, and added Xgal (30  $\mu$ l of 40mg ml<sup>-1</sup>)

Set up transformations using 5  $\mu$ l for each  $\phi$ N1 ligation mixture - left on ice for 1hr  $\rightarrow$  42°C heat shock for 30secs  $\rightarrow$  1hr phenotypic expression at 37°C  $\rightarrow$  plated out on X-gal<sup>+</sup> (30  $\mu$ l of 40mg ml<sup>-1</sup>)

Took off TLC - dried and exposed  $\phi$ N1 on x-ray film. It sounded good (as if there was good acetylation).

Diluted down "BstXI to Hha" and "Hha to BstXI" to 100ng  $\mu$ l<sup>-1</sup> and stored as working stock at -20°C. Stored remainder at -80°C.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

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Sgt. W. Kille

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David K Clarke

From Page No. \_\_\_\_\_

Checked bacterial transformants - looks encouraging - there are many colonies, quite a few Blues but also many whites.

Meanwhile spun down 0.75ml of genomic RNA(x2) - coded - died  $\rightarrow$   $-20^{\circ}\text{C}$  in preparation for RT/PCR.

Checked TCID<sub>50</sub>s (will make a final reading on Thurs) - it appears that  $32^{\circ}\text{C}$  is better for B9320 in Vero than  $37^{\circ}\text{C}$  - will await new freshly broken out Vero's before attempting to further improve titer for this virus (will use a range of the highest titer stocks for this virus at that time)

Developed TLC for 'B' rescue - looks a little confusing - will need to repeat for aesthetics - Set up 3 CAT assays - using 1 $\mu\text{L}$ , 5 $\mu\text{L}$  and 10 $\mu\text{L}$  of cell extract - let run ~ 3 hrs, then freeze away at  $-80^{\circ}\text{C}$  until tomorrow when will do the TLC.

Meanwhile set up RT/PCR RNA using BstXI to make cDNA -

Prepared 10ml of RNase A stock - very dilute - freeze away at  $-20^{\circ}\text{C}$ .

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sung W. Kibbe

Recorded by

David K. Clarke

000269

TITLE Mini Preps of Bt. RT/PCR products

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

31

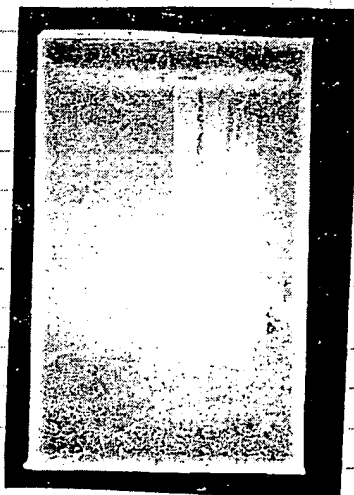
From Page No. \_\_\_\_\_

Checked growth of mini preps - Xcu/Bam are not growing as well (just as it seemed on the cloning plates - where the colonies were much smaller).  
Meanwhile prepared 1% Agarose gel to analyse RT/PCR products (BstXI to HhaI).  
Also Ethyl acetate extracted 'B' CAT assays - and equilibrated TLC tank prior to running three samples - Ran TLC and exposed O/W.

Meanwhile carried out Mini prep DNA extractions on 8 of from each group of clones (2.25K, X-B, H-X)

Didn't grow well.

Analysed all of extract at once.



Good, the 3.6K RT/PCR products are there - Probably 2 SWg in total preps - will gel purify tomorrow.

Ran half of 2.25K, and H/X

See gel overpage

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

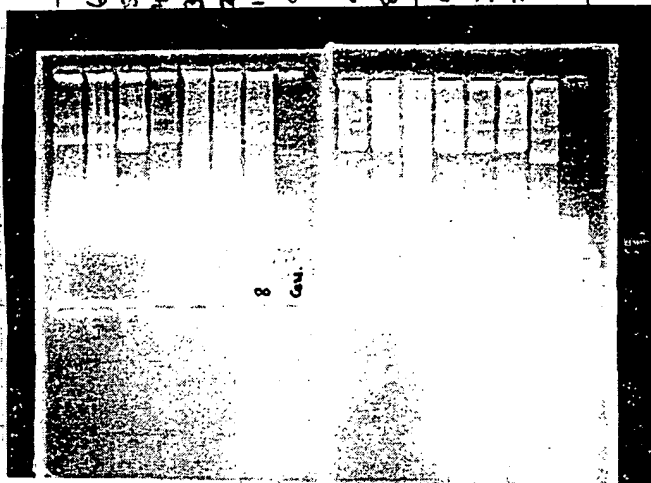
Date

*Sgt W. K. Clarke*

Recorded by

*David K Clarke*

0000270

From Page No. 31

Xca/Bum look  
good in spite of  
poor growth - 2,  
4, 8, 9

2.25K also look  
good 1, 2, 3, 4,  
6, 7, 8 all look  
+ve

Can't read the  
Hhu/Xca minis  
will remain with control tomorrow

Will set up minis 2, 3, 4, 8 for X/B tomorrow (early - poor growth?)  
1, 2, 3, 4 2.25K

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge W. Kkh

Recorded by

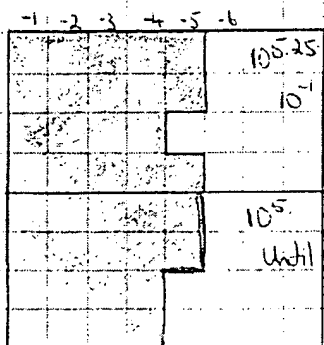
David K. Clarke

000271

From Page No. \_\_\_\_\_

Prepared 1% mini gel for analysis of H/X mini preps

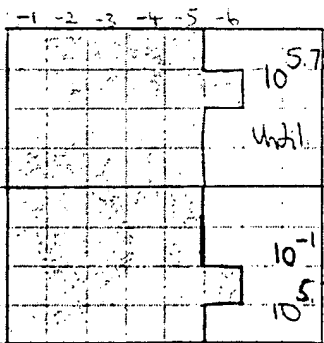
Did final reading of TCID<sub>50</sub>.



V<sub>6</sub>DC5, V<sub>1</sub>  
B9320  
37°C

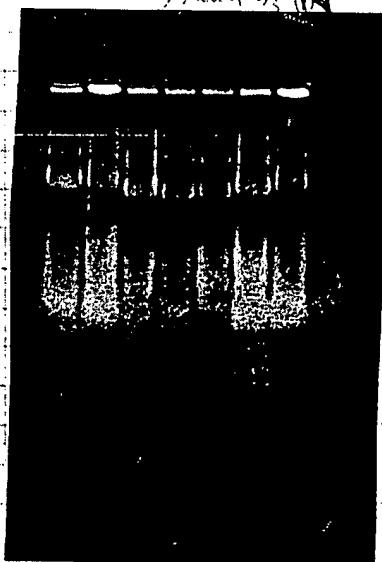
Results of TCID<sub>50</sub> indicate that 32°C is the best temp for growth of B9320

Will wait for 'New' veros before attempting to further boost titres. (will use highest titrated stocks available to try and boost.



V<sub>6</sub>DC5, V<sub>1</sub>  
B9320  
32°C

8 7 6 5  
Hlu/Xen + 2; (1) (2) (3) (4) (5) (6) (7) (8) (9) (10) (11) (12) (13) (14) (15) (16) (17) (18) (19) (20) (21) (22) (23) (24) (25) (26) (27) (28) (29) (30) (31) (32) (33) (34) (35) (36) (37) (38) (39) (40) (41) (42) (43) (44) (45) (46) (47) (48) (49) (50) (51) (52) (53) (54) (55) (56) (57) (58) (59) (60) (61) (62) (63) (64) (65) (66) (67) (68) (69) (70) (71) (72) (73) (74) (75) (76) (77) (78) (79) (80) (81) (82) (83) (84) (85) (86) (87) (88) (89) (90) (91) (92) (93) (94) (95) (96) (97) (98) (99) (100)



Unfortunately doesn't seem there are any clones containing the correct inserts, although will check 1, 7, and 8. - Will also look at 10 more clones

It is possible that the fainter band in the (on) is the one from which case 1, 7, and 8 would be correct. (2-3K insert)

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Recorded by


Sgt. W. L. L.

Peniel K. Clarke

000272

جزء 3 - 2

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Origin

000273

From Page No. \_\_\_\_\_

Gel purified 3.6kb BstII to Hha fragment - phenol chloroform extracted  
and ethanol ppt'd with glycogen

Set up the following O/S mini preps

Xba/Bam 2, 4

2.25K frag 2, 4

Hha/Xba 1, 8

} for digestion

Also set up 11-18 for uncut analysis Hha/Xba

Developed 'B' Resene cut Assay - it looks very good. It appears to  
be ~ 20 fold better (see antiserum).

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sgt. W. Koble

Recorded by

Wm. K. Clarke

000274



From Page No. \_\_\_\_\_

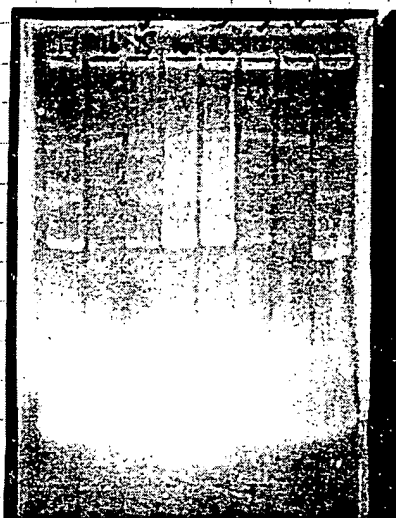
Carried out Mini prep DNA extractions on clones 11-18 for  
 Hha/Xca to identify further clones with inserts

Kpn I/Xho I cut 2.25K  
 clones

Kpn I/Xho I cut Xca to Bam  
 clones (2.6K)

Kpn I/Not I cut Hha to Xca  
 clones (2.3K)

Saved 1/2 of mini-prep material  
 for further digestions



H/X clones 11, 12, 13, and 15  
 all look positive.

Also did double digests on  
 2.25, X/B, and H/X clones  
 identified as positives  
 yesterday

Meanwhile prepared L-Agar plates (Amp<sup>+</sup>) to restreak clone banks (Tomomaru)  
 2.25K, Xca/Bam, Hha/Xca

looks as though the  
 H/Xca inserts are not  
 correct - will try  
 11, 12, and 13 with  
 Kpn I/Not I - will check  
 some more for presence of  
 insert



Both the 2.25K and Xca/Bam inserts  
 look good - correct size as  
 predicted

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sgt W. K. Kuhl*

Recorded by

*David K. Clarke*

From Page No. \_\_\_\_\_

Checked PCR II Vector for F<sub>1</sub> Ori - it's there - so will be able to use that plasmid for the site directed mutagenesis.

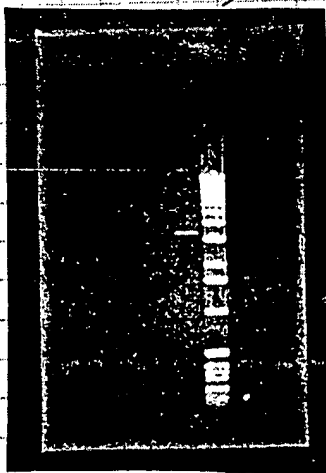
prepared 1% agarose gel for analysis of purified 3.6K fragment - also

set up RT/PCR x 2 on Hha/Xba fragment - modified the annealing temp to 45°C for one RXN, left at 37°C for the other.

\* Will cut Xba/Bam clone with EcoRI/Pst.I to give Vector + 1500bp  
700bp  
400bp

\* Will cut 2.25K clone with EcoRI/HindIII to give Vector + ~1800bp  
~1200bp

Restructured clone  
BamKs 3K, 1.5K, CAT/RSV  
put at 37°C o/n.



Good the 3.5K frag looks fine  
and should have ~ 50ng total remaining.  
Will ligate that into 2nd of Vector.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suz W. White

Recorded by

Daniel K. Clarke

0000276

TITLE: Analysis of H/Xen RT/PCR products / Origin of BstXI / HhaI into PCR II

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

37

From Page No. \_\_\_\_\_

Prepared 1% Agarose gel for analysis of RT/PCR products  
Chloroform extracted and EtBr stained with glycogen

Set up 2/3 mini preps 1, 12, 13 H/X for digestion (Kpn/Not I)  
Also set up 7 more minis for preliminary test

Set up 2nd ligation - All of remaining BstXI-Xba fragment with  
2nd (5' end) of Vector in 20 µl vol. 12°C env. Also  
set up a No insert control

Qualities required for 'L'-end Oligos

- ① T7 promoter flush with precise end of RSV genome - get from CAT-RSV construct.
- ② should contain 2-5 restriction sites to facilitate modification and/or cloning.
- ③

Qualities required for 'N'-end Oligos

- ① should give precise run off transcripts - include Hya I
- ② should include 2-5 restriction sites not in genome to facilitate further modification and/or movement between vectors.

PCR up small fragment at L-end - using Sca I / Sph I / Xba I sites  
+ end oligo - ligate to synthesized ds DNA containing promoter + restriction sites  
and a 1 base overhang (T) - then end clone - use Hind III site at end of  
desired restriction sites to clone back into PCR II containing Not I 3' clone.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

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Date

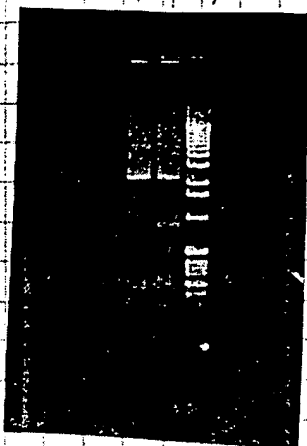
Suzi W. Kuhl

Recorded by

Harold K Clarke

0000277

From Page No. 57



Good H<sub>2</sub>O/X<sub>2</sub>O RI/RI products contain  
2.2g for both samples - Total = 2.2g

The 45°C annealing temp looks  
better than the 37°C, with a lot less  
low m.w. non-specific product

Will combine both and electro elute  
purify on trees

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kibbe*

Recorded by

*Daniel K. Clarke*

TITLE Transformation with BstXI/Hinc

From Page No.\_\_\_\_

Carried out mini-props on H<sub>2</sub>/Xen 11, 12, 13, 18, 19, 20, 21, 22, 23, 24

KpnI / NotI digested

Did bacterial transformations with 5  $\mu$ l of O/N ligation mixtures.  $\rightarrow$  O/N 37°C



19 and 22 look like the ones that  
I want - will extract more  
DNA for Digestion later today

Proceeded with extraction and phenol:chloroform extraction - left in EtOH until tomorrow (will then do KpnI/NciI/RNase digestions)

Completed double digests on 11, 12, 13 and analysed the products on a 1% gel

No - there are no clones containing inserts of the current size.

**To Page No.\_\_\_\_\_**

Witnessed & Understood by me,

Boyd W. Kille

Date \_\_\_\_\_

**Invented by**

Recorded by \_\_\_\_\_

Recorded by Dwight K. Clarke

**Date**

From Page No. \_\_\_\_\_

Checked clones - looks v. good - control plate is showing predominantly blue colonies, while Bst/Hha are showing predominantly white colonies.

Set up restriction analysis on H/X 22 and 19.

KpnI/NotI.

prepared 1% agarose gel for analysis of digestion products.

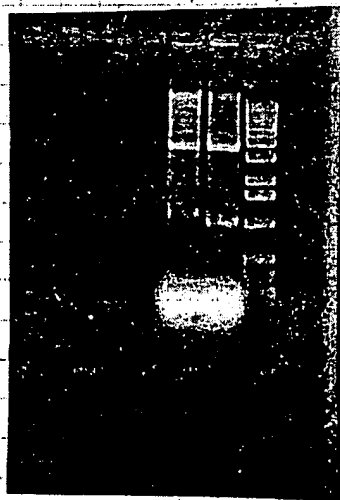
Also prepared a 1% gel for purification of Hha/Xba fragment.

Set up some minis of potential Bst-Hha clones and subcloned them onto master plates.

Completed purification of New Hha/Xba fragment.

Phenol Chloroform extracted

and EtOH pptd with glycogen.



19 and 22 are not the correct clones either - both are ~100 bases shorter than what I am looking for.

Will look at single digest products next time to make sure there are no internal KpnI or NotI sites.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Serge W. Khali*

Recorded by

*David K. Clarke*

TITLE Mini preps on Potential BstXI/Hha clones

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

41

From Page No. \_\_\_\_\_

Carried out mini preps on BstXI/Hha clones 1-7.

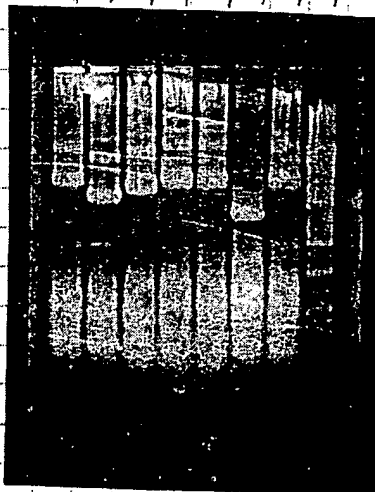
loaded  $\frac{1}{4}$  on a gel for a relative comparison (asps. forgot no-insert control)

Also digested  $\frac{3}{4}$  of each with EcoRI/Rouse A. ~1.5-2 hrs at 37°C

Also checked purified Hha/Xba fragment in a gel to check conc. etc.

looked at  $\frac{1}{5}$  (2  $\mu$ l out of 10)

Hha/Xba  
1000 ladder



1, 3, 4, 7 look as though they could be positives

Will check their EcoRI restriction pattern

Also checked yield from Hha/Xba purification - looks pretty good and fairly pure - Used  $\frac{1}{4}$   $\mu$ l in ligation with 2  $\mu$ l Vector - 12°C on

Prepared yet another 1% Agarose gel to analyse EcoRI digests - they look good (see overpage)

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

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Date

Suzanne W. Kuhl

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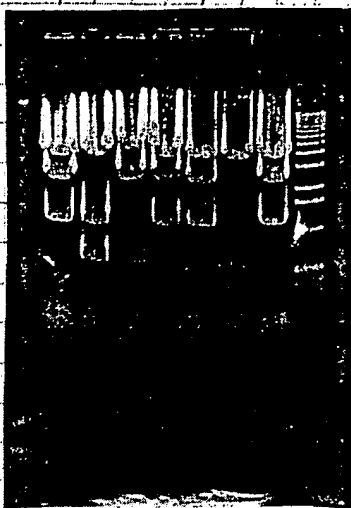
David K. Clarke

000281

Project No. \_\_\_\_\_

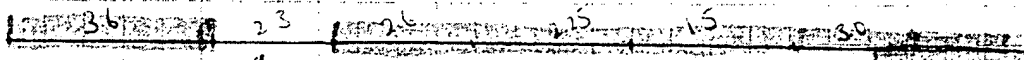
Book No. \_\_\_\_\_ TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_



1, 3, 4, 7 are indeed correct  
good! now have all but the 2, 3, 6.  
they closed (see below)

prepared a fresh stock of  
Ramp. Sargent!



To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sgt. W. K. Kille*

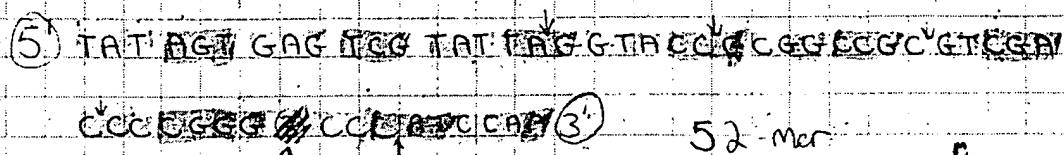
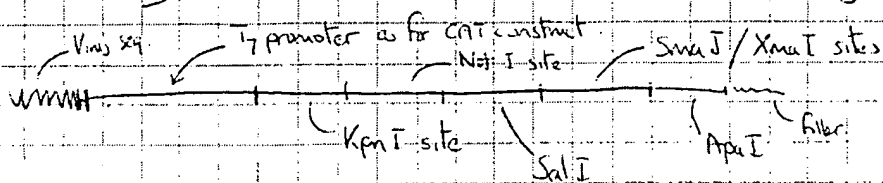
Recorded by

*David K. Clarke*

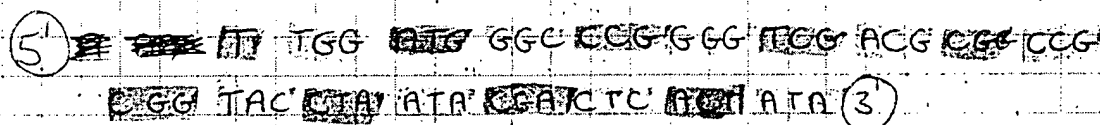


From Page No.\_\_\_\_

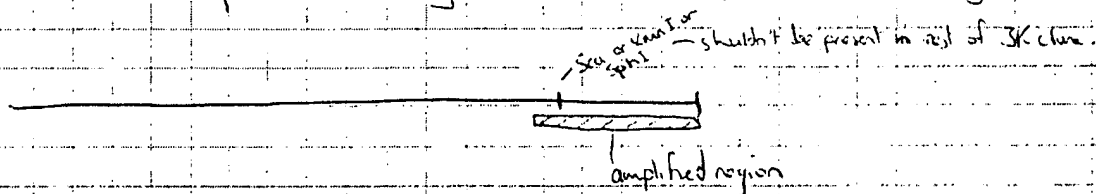
Prepared Oligos for modification of the 'Vims' 5' and (L-gene)



Complementary Oligo



Will PCR up a small fragment of the end portion of the L gene



primers will not be  
kinased  $\therefore$  no S(P)  
So will not self ligate.

will then ligate both pieces of DNA together (only one of the synthetic oligos will be kinased) and treat with restriction enzymes and relax into 3K clone appropriately cut to accept the fragment

need to check <sup>3K</sup> plane

concentration with initial double digest

**Witnessed & Understood by me,**

**Date****Invented by****Date**

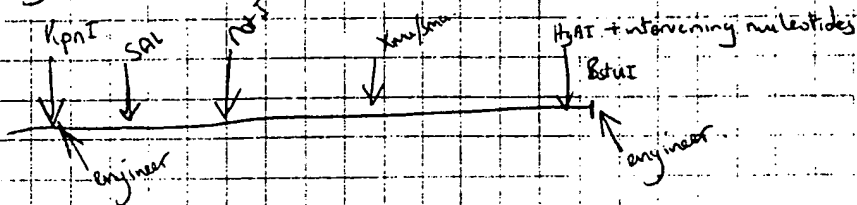
To Page No. ....

Recorded by \_\_\_\_\_

Recorded by  
David K. Clarke

From Page No. 43

For the 3' end of the Vms will use synthetic Oligos, with an engineered BstXI, HgAI and KpnI, NotI, SalI, XbaI



Need to check orientation of 3.6K clones to find one with the currently positioned (on the <sup>current</sup> right side)

Will ligate synthetic DNA into BstXI/KpnI cut construct (Syn DNA will lack 5' phosphates so will not self ligate)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sgt. W. K. Kuhl

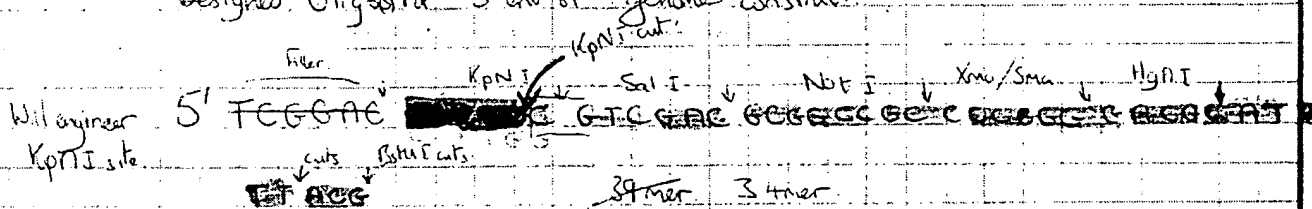
Recorded by

David X. Clarke

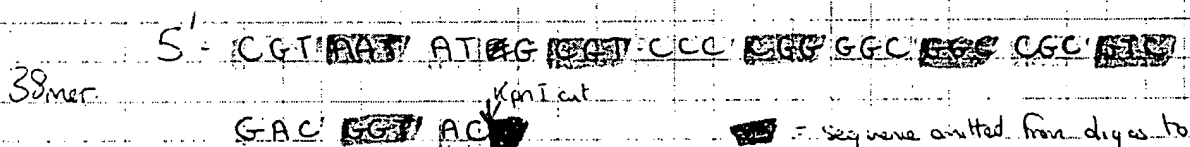
From Page No. \_\_\_\_\_

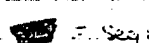
Checked bacterial transformants - there are significantly more whites vs control with the 2.3K RI/PCR product. - let grow for another few hours - then set up O/N minus (will wait to set up master from the O/N cultures). - will do 14 initially.

Designed Oligos for 3' end of genome construct



The complement



 = sequence omitted from digest to accommodate KpnI ends

Xba to Bam clone will contain the non-translated region of 'F' where a mutation can be introduced to give a restriction site.

Pme I can be introduced at ~ position 7420 (see Book) will sequence around this region from the translated portion to verify sequence. Will need to do large scale pops of Xba/Bam to get plasmid for sequencing.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kuhl*

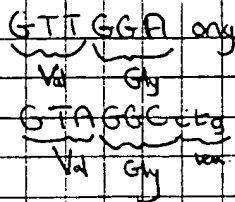
Recorded by

*David K Clarke*

0000285

From Page No. 45

Can also introduce a StuI site  $AGG \downarrow CCT$  in the hydrophobic domain of the F' protein without altering the coding capacity at position ~ 7292. <sup>Anchor</sup>



Set up 14 O/N minus — will extract DNA tomorrow and do a preliminary gel analysis.

Witnessed &amp; Understood by me

Date

Invented by

Date

To Page No. \_\_\_\_\_

*Steve W. White*

Recorded by

*David K. Clarke*

TITLE \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

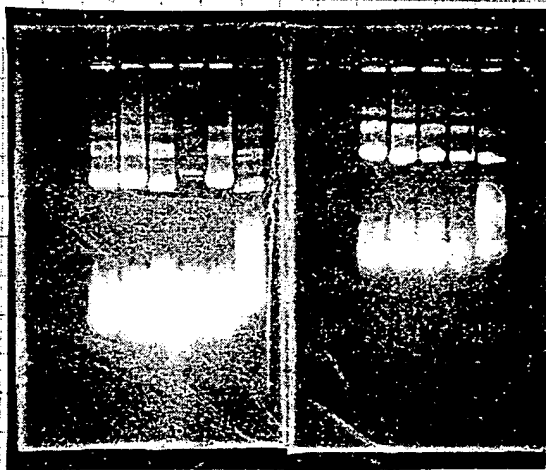
47

From Page No. \_\_\_\_\_

Went through mini preps in search of 2.3K Hha/Xba clone  
≈ 5 of the mini preps either hadn't grown at all, or only very weakly  
Extracted DNA from the other 7

Prepared 2 x 1% Agarose gels and ran out minis with Pst II control plasmid.

However - will  
try another 14  
mini preps just  
to make sure.



It doesn't look as  
though there are any  
clones of the correct size

This indicates that either  
the SH, or G protein are  
toxic - will have to devise  
a new scheme to clone  
this area

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kuhl*

Recorded by

*Wendell K. Clarke*

0000287

From Page No. \_\_\_\_\_

Set up ten more mini preps to check for potential 2.3K (Hha/Xba) clones

Also set up PCR reaction on remaining purified Hha/Xba DNA

6ul MgCl<sub>2</sub>

8ul x 10 Reaction buffer

2ul each dNTP

2ul each primer

H<sub>2</sub>O to = 80ul

0.6ul Taq

15°C annealing temp.  
30 cycles

There is a unique AvcII site at position 2129 (towards the end of the 'N' gene)

can use this to make cDNA → XhoI site at position 4481

May be able to make a cDNA clone, then from PSET XhoI to either PFI or Eum1105.I - depending on

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Lucy W. Kuhl*

Recorded by

*David K. Clarke*

TITLE Testing Mini Preps for 2.3K insert.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

49

From Page No. \_\_\_\_\_

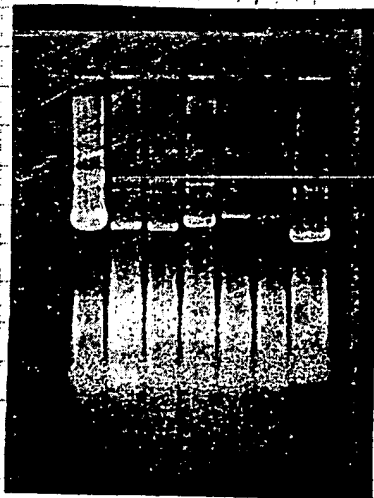
Run out a sample of Hha/X amplified DNA to assess  
amount of amplification - Not v good - little amplification occurred  
Tossed sample - will try RT-PCR using new primers etc

Invitrogen One-shot host is EcoK but Dam<sup>+</sup>/Dcm<sup>+</sup>

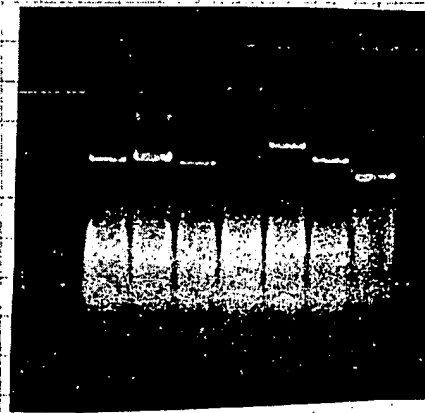
Will need to use Eam 1105 I - will check availability

tanawan

Meanwhile carried out mini prep DNA extractions on  
12 more potential Hha/Xca clones - Run  $\frac{1}{4}$  of each prep  
on a  $\frac{1}{2}$  Agarose gel - freeze the remaining  $\frac{3}{4}$  at  $-20^{\circ}\text{C}$



Will cut 1, 10, 25, 26, 27 with  
Kpn - look at  $\frac{1}{3}$  + Not I (RNotA)



To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

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Date

Suzanne K. Kille

Recorded by

Wendy K. Clarke

0000289

From Page No. \_\_\_\_\_

From P \_\_\_\_\_

Back to work!

Phenol - Chloroform extracted mini preps 1, 10, 25, 26, 27 and E. coli potted

Then set up Kpn I digestions - Same as 1 hr at 37°C

Prepared 1% Agarose gel for analysis of 1<sup>st</sup> cut.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

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WI

Steve W. Kihl

Recorded by

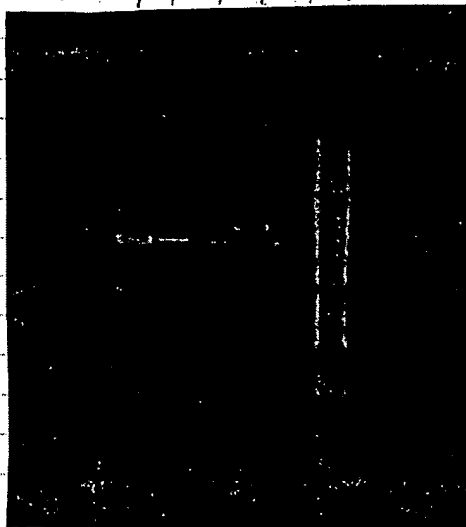
David K. Chishe



From Page No. \_\_\_\_\_

Set up 1% Agarose gel and analysed Kpn.I digests of potential 2.3K clones (Plasmid cloneform extracted the bulk of the DNA and EtOH ppt<sup>d</sup> prior to cutting with Not.I)  
<sup>27, 26, 25, 10, 1</sup> ladder

Bst 11071 is an  
 Isoschizomer of Xca.I



It is clear that even though CCC plasmid migrate the same distance they may contain different sized inserts

10 looks as though it is the correct size ~ 6Kb to contain the 2.3K insert.

Infected Vero TC25 with a variety of B9320 stocks (to boost higher titer stocks)

① V<sub>6</sub> DC5, V<sub>1</sub> 32°C → Dil at 32°C  
 → Undil

② V<sub>6</sub> DC5, 32°C → Dil 32°C  
 → Undil

③ V<sub>5</sub> undil → Dil 32°C  
 → Undil

Used 0.25 ml of inoculum for each TC25 and adsorbed for ~3 hrs at 37°C - then placed at 32°C

To Page No. \_\_\_\_\_

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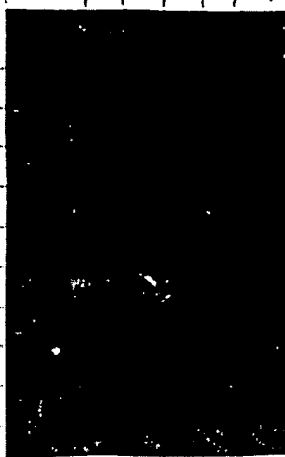
*Suzanne W. Kish*

Recorded by

*David K. Clarke*

000291

From Page No. \_\_\_\_\_



Number 10 looks like it <sup>should</sup> be the  
one. The insert size is correct.  
2.3Kb. as is the regenerated.

Will set up a fresh *gyn* culture  
to double check - will also streak out  
on a separate plate as a reserve

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Serge W. Kibler*

Recorded by

*David K. Clarke*

TITLE: Checking Cbe 10 for Hha/Xba Insert

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

53

From Page No. \_\_\_\_\_

Carried out mini prep <sup>DNA</sup> extraction on clone 10 - froze down X3 aliquots of this DNA culture in 15% glycerol, to act as a -80°C stock.

Set up Bst 1107 I digestion on mini prep DNA in 40µl final vol; incubated at 37°C (usual for *B. stearothermophilus*) for 1-2 hrs - used 1µl of enzyme. Then phenol:chloroform extracted and EtOH pptd.

Checked c.p.e. on infected Vero's - very little sign of c.p.e., but will check again later today.

Proceeded to set up Sac I digestion - (while retaining a sample of Bst 1107 I cut DNA for gel analysis) - in 30µl final vol.; 37°C 1µl Enz.

NB Strategy for RSV vaccine production, using naked DNA

1) Put the CMV promoter just downstream of the entire cloned RSV genome (cDNA) - multiple engineer in splice sites between genes to give monocistronic messages OR put a CMV promoter in the genome between each gene to give individual mRNA synthesis from each promoter.

Ordered the 4 Oligos necessary for modification of the 3' and 5' ends of the RSV genome cDNA.

Rechecked c.p.e. which are now becoming more apparent.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Suzanne W. Kuhl

Recorded by

David K. Clarke

000293

54

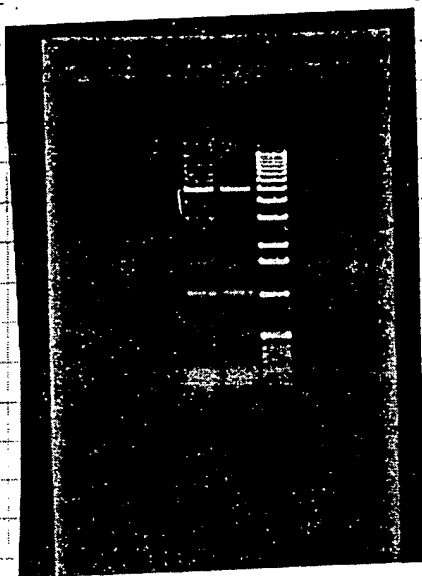
Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

BT 11075 - SMC  
BT 11075  
- 606



Restriction pattern indicates  
that c.b.a. 10 is NOT what  
we want. - I should not have  
2-Bst-11075 sites

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sgt. W. Kble*

Recorded by

*David K. Clark*

0000294

TITLE: RT/PCR of Xho/Xca region of RSV.

From Page No. \_\_\_\_\_

Prepared L-Agar for transformation later today. (Need 30°C incubator)

Set up RT/PCR rxn using XhoI to Xca primer to try and  
(XhoI to Bam)  
split up the clone that's causing problems. Also ran a rxn for PsmuK  
to try and improve her cloning efficiency.

RT/PCR product should be 1.35Kb

Electroporated some E. coli (George's) with 1 µl of ligated 2.3K fragment  
with RRII (1.8KV) at 30°C.

Carried out phenotypic expression to minimise recombination etc.

3-40 to 4-40pm - plated out and put 2 at 30°C and 1 at

37°C

Harvested 'B' 9320 virus infections and froze at -80°C - will  
run TCID<sub>50</sub>s on Monday

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Ibbel*

Recorded by

*David K. Clarke*

From Page No. \_\_\_\_\_

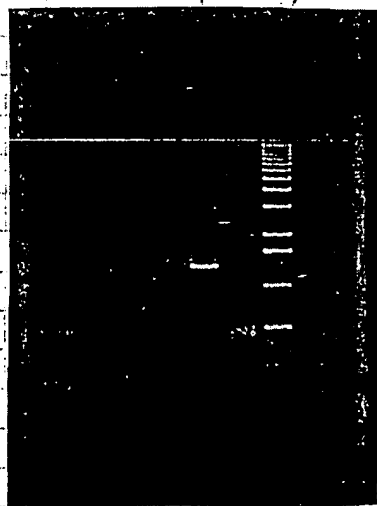
Prepared 1% Agarose gel for analysis of RT/PCR products - Run out IKbladder, Pannuk's RND, and mine

Also checked electroporation clones - there are quite a few & whites at 37°C - streaked out ~36 and grew o/n at 37°C as a master plate

Also checked clones incubated at 30°C - there are quite a few there too - the colonies are smaller and blue colour has not yet developed - may incubate for a further 24 hrs or may plate out on a master-plate later today.

streaked out clones grown at 30°C → 30°C master plate

Will set up Minis tomorrow



Good the Xho/Xen frag

looks perfect - at the correct position and in good abundance

Pannuk's RND did not work

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Invented by

Date

*Serge W. Kibbe*

Recorded by

*Daniel K. Clarke*

000296

TITLE: Gel purification of Xho/Xba RT/PCR product

From Page No. \_\_\_\_\_

Prepared 1% Agarose gel for purification of Xho/Xba RT/PCR products - ran gel and proceeded with electrophoresis of the DNA band - everything went well - Phenol/chloroform extracted and etOH ppt<sup>d</sup> with glycogen carrier - will set up o/n ligation tomorrow. checked on growth of master plates - interestingly the clones grown at 30°C showed more uniform and stronger growth than those growing at 37°C - also on the original plates the lack of blue color expression at 30°C (even following o/n at 4°C) indicates that there may be no expression from the lac-Z region of the transforming plasmid (this region contains the RSV cDNA insert).

To Page No. \_\_\_\_\_

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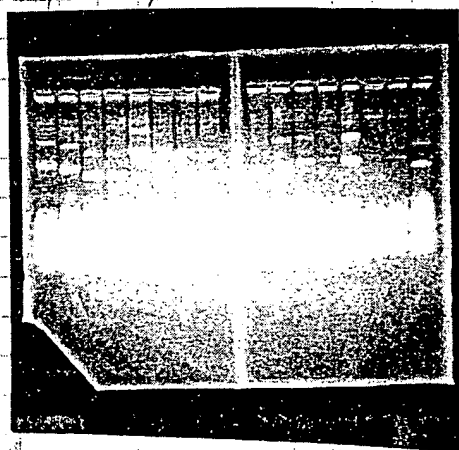
Suz W. Khl

Recorded by

David R. Clarke

From Page No. \_\_\_\_\_

Carried out mini prep DNA extractions on clones grown at 30° and 37°C - Prepared 2 x 1% Agarose gel for 1st look analysis.  
 loaded 10<sub>10</sub> (of 40) for each sample



Will take a look at Nos 6 and 10. but neither look v. promising

phenol chloroform extracted these samples and etOH pptd - prior to digestion and gel analysis.

Set up O/N ligations of purified Xho/Xcu - used 1/3 of total purified material  
 also set up control ligation

Carried out T.C.I.D.<sub>50</sub>s on V<sub>6</sub> 32°C B9320.

V<sub>6</sub> DC5, V<sub>1</sub> B9320 32°

V<sub>6</sub> DC5, V<sub>2</sub> B9320 32°

Set up O/N mini preps of the two 'end' clones. 3Kb NCTAI at 5' (Lent)  
 3.6Kb Bsty/Hha at 3' (New)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Suz W. Kibbe*

Recorded by

*David K. Clarke*



Transformation with Xho/Xca  
TITLE: Digestion of Miniprep DNA - potential 2.3K Hb./Xca

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

59

From Page No. \_\_\_\_\_

Set up Bst 1107.I digestions on (6) and (10) from yesterday  
prepared 1% agarose gel for analysis

Called BRL about plasmid/clon stability - they said HR101 performed best  
and to use rich broth (Ternic broth, SOC media/SOC agar) and not to grow  
cells past logarithmic phase

Called Invitrogen - They suggested using Top 10 or DH10 Hosts! - not very  
knowledgeable

Called Clontech - Not much help - may get back to me

Called Promega - Rick IX 1360 - suggested JM109 and recommended  
Stratagene (Sma-clon strains)

Completed double digest on (6) and (10) with Xho I - run on gel for  
analysis

Also carried out transformation with Xho/Xca RT/PCR product + Control

Replated Bstn/Hb. bugs in a fresh agar plate to keep viable - grows  
O/N at 37°C

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Lyne W. Koble*

Recorded by

*David H. Clarke*

000299

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_ TITLE \_\_\_\_\_

From Page No. 59



unint. planned

These clues are not correct  
either. (There are some  
sort of rearrangements  
going on)

Will wait and see what  
xho/xca clues bring.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne W. Kibler

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David K. Clarke

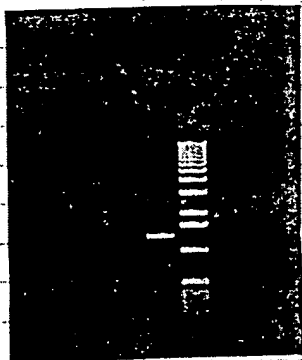
0000300

From Page No. \_\_\_\_\_

checked transformants - not a ben - <sup>no white colonies</sup> indicates G-protein is highly toxic - may be the problem!

Called Stratagene (Brian) - suggested using 'SURE'-strain which minimises recombination events and/or 'Able C' and 'Able K' - probably should not use Blue colour selection since it will induce expression from the lac promoter - reduce plasmid copy number/expression.

Started plasmid preps - Qiagen



linked xho/xen fragment

This gel proves that there was an abundance of DNA in the ligation with PCR II vector

∴ this region must indeed be very toxic to bacteria (it contains the entire coding sequence of the 'G' protein)

Things to try

- 1) Able C, K, Sure cells (strains)  $\frac{+}{-}$  blue selection  $\frac{+}{-}$  Rich medium and Xen/Ben
- 2) RT/AR Hha/Xen again and ligate to 3.6K clone appropriately cut. - then clone large fragment
- 3) Clone fragment in Yeast
- 4) Cut Hha/Xen fragment with Xho - try cloning both fragments
- 5) Clone Hha/Xen into pBR322  $\rightarrow$  HB101 or other strain

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sign W. Koble

Recorded by

David K Clarke

From Page No. \_\_\_\_\_

Will cut the 3K clone with Sac I (has 2 sites - internal) and one in the plasmid. - 3 total

Will cut 3.6K clone with Eco RI (one internal site - two flanking sites)

Will also ligate some <sup>PCR II</sup> vector with Xho I and the small amount of remaining purified Hha/Xba fragment

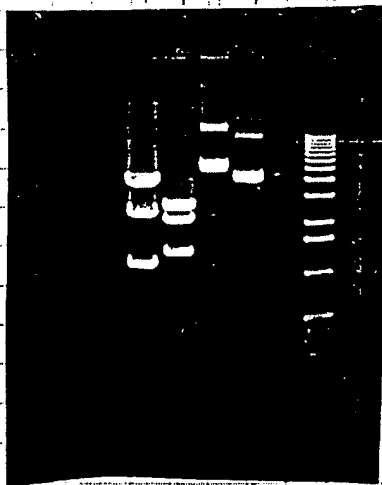
Prepared 1/2 gel and ran out digests

LHS. ladder / Xho / Hha/Xba / uncut 3K / uncut 3.6K / Sac cut 3K / Eco cut 3.6K

*3.6K Eco cut*  
*3K Sac cut*  
*3.6K clone*  
*3K clone*  
*ladder*

There was not enough DNA to see on the Hha/Xba Xho digestion

Plasmid cloneform extracted. Xho cut PCR II - ppted with glycogen



Good - plasmid prep. look fine and the Sac and Eco sites are as expected

Wrong! 3K clone is unstable and has undergone deletion of part of the insert or plasmid

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sup. W. K. Kibbe*

Recorded by

*David K. Clarke*

TITLE: Quantitation of Purified Oligos

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

63

From Page No. \_\_\_\_\_

It appears that the 3K clone has undergone a 1K deletion - need to go back and check minus from plates - if those are also deleted, then will have to transform from original minus into 'SURE' strains

Meanwhile dried down purified oligos from 50% ethanol/methanol - resuspended in  $H_2O$  and measured O.D.

One of the Oligos had not been purified correctly - redissolved large white pellet in 3ml  $H_2O$  and loaded onto column - eluted - dried down.

Stored dried oligos at  $-20^\circ$  prior to dissolving in  $H_2O$

Did a preliminary read on TCID<sub>50</sub>s - does not appear to be any Stellar B' virus stocks - will check again on Tues for final read.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sgt. W. Kille*

Recorded by

*David K Clarke*

000303

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Back from San Diego weekend!!  
Final TCID<sub>50</sub> Readings on B-view stocks

From Page No. \_\_\_\_\_

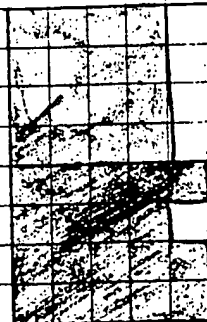
Made final TCID<sub>50</sub> Readings on B-view stocksB9320 V<sub>6</sub> DC5, V<sub>1</sub>10<sup>-1</sup>  
(32°C)

Undil

10<sup>-1</sup>  
B9320 V<sub>6</sub> DC5, V<sub>2</sub>

(32°C)

Undil

(10<sup>-1</sup>)B9320 V<sub>6</sub> 32°C

(Undil)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

Sgt. W. Kibbe

Daniel K. Clarke

0000304

TITLE: Lab Bacterial Transformation ('SURE', 'ABLE' cells)

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

65

From Page No. \_\_\_\_\_

prepared 300ml L-Agar for transformation of 'SURE', 'C-ABLE', 'K-ABLE'  
and 3K DNA

Redissolved purified Oligos in 200µl H<sub>2</sub>O and measured the O.D.<sub>260</sub>  
(used 10µl diluted into 490µl H<sub>2</sub>O) - Yields look good 0.7-2 µg µl<sup>-1</sup> for  
each

Set up the following transformations.

~2µl of Hha/Xba (2.3K) ligation mixture	each into	ABLE C	} up of both for "Trans"
		"K"	
~1µl of NE13 (3.3K) mini prep DNA	into	ABLE C	
		"K"	
1µl of 'CON' pUC18 in 50µl each		SURE	
	of ABLE C		
	ABLE K		
	SURE		

Set up O/N mini preps of 1.5K clones ~~2~~ only.  
2.25K clones x2 (Bam/Pml?)  
Xba/Bam clones x2.

Have 3.6 K clone grown up - it is okay  
Am transforming with 3K clone - so will not do O/N minis on that one.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Suzanne W. Koble

Recorded by

David K. Clarke

000305

From Page No. \_\_\_\_\_

Checked bacterial transformations (Sure - Able C - Able K) - Efficiency with Able C' and 'K' are lower than with 'SURE' - however did get a few transformations on 'C' and 'K' with 2.3K (Hha/Xca) ligat<sup>n</sup> mixture - many more clones on 'Sure' - will set up 2 mini preps O/N

Meanwhile did mini preps on 2.25K clones X/Bam clones - left 1.5K mini prep to grow longer - (1.5K did grow finally and carried out mini prep DNA extraction)

Infected 10 x TC 150 each with 1ul of  $V_{3}H_1$  virus stock - absorbed  $\approx$  3 hrs at 37°C, then added growth medium and incubated at 32°C

Set up Digestions on mini-prep DNAs XcaI/BamHI and 2.25K clone

① Bam HI on each ~15 hrs

Will look for 2.25K or 2.6K

② Either SptI or Bst 1107I ~2 hrs

Proceeded to analyse the products on a 1% Agarose gel.



Good, both clones for each of 'Xca/Bam' and '2.25K' clones contain the appropriate sized inserts (+ restriction sites)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Serge W. Kibbe*

Recorded by

*Harold K. Clarke*



TITLE Mini prep analysis of potential 2.3K clones.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

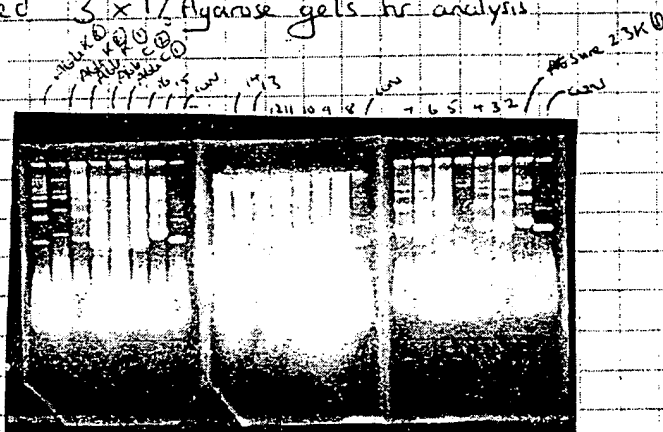
67

From Page No. \_\_\_\_\_

Prepared L-Agar - Amp plates to restreak clone banks.

Then proceed with mini preps for potential 2.3K clones in 'Sure' 'Able' etc.

prepared 3 x 1% Agarose gels for analysis



Will take a look at  
Able K ② by double  
digestion; but  
unfortunately it doesn't  
look as though any  
2.3K clones were  
obtained - even with  
the 'Sure' - Able C/K systems.

Restreaked entire clone bank - will check again tomorrow and store at  
4°C if they are fully grown.

- ① Use HB101/pBR322 to clone this fragment.
- ② Use Yeast
- ③ Ligate the RT/PCR product to the cloned DNA on either side and then try cloning.

Hep-2 cells are showing nice CPE clearly at 24h p.i. Will harvest  
as early as possible tomorrow morning.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Steve W. Kelle*

Recorded by

*David K. Clarke*

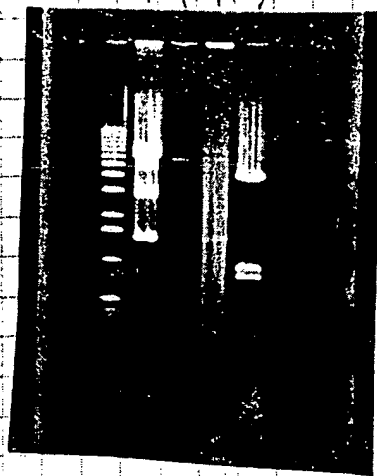
000307

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_



Need to check  
3K 'Sure' with EcoRI and  
Bam HI.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Serge W. Khle*

Recorded by

*David K. Clarke*

0000308

TITLE Nucleocapsid Preparation

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

69

From Page No. \_\_\_\_\_

C-pe on infected Heps was v. extensive - initiated Nucleocapsid prep at 9am - let the Nucleocapsid pellet resuspend on ice prior to loading onto K<sup>+</sup> tartrate gradients.

Ran only 2 gradients this time.

Also carried out minipreps on remaining SURE clones (forgot control on gel).

Ran on a 1% Agarose gel.

Carried out digestions of "3K in SURE" (Bam HI and Eco RI separately)

Also did an Eco RI on Abbe K ② (Page 67)

All 'SURE' minipreps showed plasmid of the same size - Didn't have 'CUN' so can't tell if there is an insert - re run one sample with 'CUN' to check again.

SURE 2.3

PCR on one sample with 'CUN' to check again  
EcoRI Abbe K ②  
BamHI SURE 2.3  
EcoRI 3K SURE  
1Kb ladder

Will streak out '3K SURE'

on an Amp<sup>r</sup> plate to have a stock.

The insert size of both 'Abbe K ②' and SURE 2.3 is too small.

3K 'SURE' does appear to contain the correct insert (after Eco RI digest). However the correct fragments are running slightly faster/further than anticipated - this may be due to the fact that the digests were loaded directly onto the gel without dilution etc in electrophoresis buffer.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Suzanne W. Kibbe

Recorded by

David K. Clarke

000309

From Page No. \_\_\_\_\_

Took off nucleocapsid spin - bands look good on both gradients - Harvested into 4 eppys - diluted and spin down in microfuge  
Rinsed with T.E., then resuspended each pellet in 500  $\mu$ l TE, 50  $\mu$ l of stock Prot K, and 50  $\mu$ l of 20% SDS - Incubated at 37 $^{\circ}$ C for ~ 5 min, then phenol chloroform extracted x2 - EtOH ppt<sup>d</sup> - yield looks pretty good

Infected Vero cells with B320 V<sub>D</sub>CSV, 10<sup>-1</sup> 32 $^{\circ}$ C - DI/undil at 32/37 $^{\circ}$ C

Absorbed ~ 3 hrs - then added G. medium.

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sgt. W. K. Clarke*

Recorded by

*David K. Clarke*

To Page No. \_\_\_\_\_

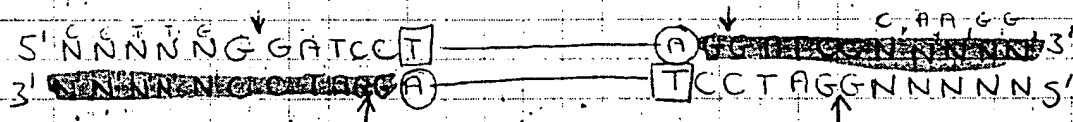
From Page No. \_\_\_\_\_

Called Jericho Security!

Prepared fresh L.B. Agar Amp<sup>r</sup> for transformation (+2°C water bath)  
 Set up transformation with 5 µl of DNA from cov(pB322) and my H<sub>h</sub>/X<sub>h</sub> ligation mixture.

Also set up Bam HI digest on 3K SURE - left ~1 hr at 37°C  
 then phenol chloroform extracted - ethanol ppt<sup>ed</sup> in preparation for Xho I digestion  
 Checked c.p.e. on Vectors infected with B9320 - 37°C show some c.p.e. but little is visible at 32°C (will check again later today).

Designed Bam HI linkers



~~These~~ These Oligos are Kinased

Set up Xho I digest ~1 hr for ~1 hr - prepared a 1% Agarose gel  
 and run to analyse digestion products <sup>Bam/Xho cut 3K SURE</sup> ladder

3K Sure is OKay

Good. Insert look believably to be 2.97 Kb as predicted and expected (but may be a small deletion in the vector). Will try a larger scale prep soon.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Aye W. Kable

Date

Invented by

Recorded by

Daniel K. Clarke.

Date

000311

From Page No. \_\_\_\_\_

Checked transformants - there are plenty on the <sup>Hba</sup>Xca plates - will select 14 for mini preps.

Checked c.p.e. on B9320-infected Vero cells - they are V advanced at 37°C until (complete fusion of monolayer) - less c.p.e. at until 37°C and still less at 32°C Diff. until

Ordered the Bam HI linkers (from overpage) - linker ② should be kinased

$$7 \times 4 = 28^\circ\text{C}$$

$$4 \times 2 = 8$$

$\frac{36^\circ\text{C}}{36^\circ\text{C}}$  melting temp = 36°C - Hold at 37°C for 30 min - 32°C for 30 min

27°C for 20 min 22°C for 20 min

\* Infected six mice with B9320 V<sub>6</sub>DC5, V<sub>1</sub> (10<sup>-1</sup>) 32°C - Used 100 µl of virus (titre ~ 10<sup>6</sup> pfu/ml<sup>-1</sup>) intranasal (Anesthetized mice first) Also injected <sup>of the same</sup> 2 mice in the leg with some remaining virus.

Meanwhile harvested B9320 infected Vero's

32°C 10<sup>-1</sup> until

37°C 10<sup>-1</sup> until

} now V<sub>6</sub>DC5, V<sub>2</sub> 4-25-94

Set up 14 mini preps to check the <sup>Xca</sup>Hba construct in HB101. (Also) did 2 pBR322 minis for control (4.33 kb)

To Page No. \_\_\_\_\_

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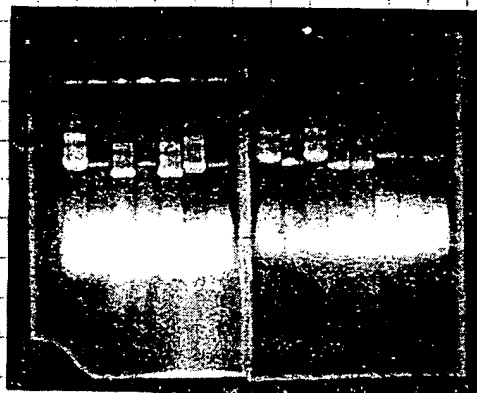
David A. Clarke

From Page No. \_\_\_\_\_

Carried out mini-prep DNA extractions on potential Hha/Xba clones in  
HB101

Prepare 2 x 1% Agarose gel for the 1<sup>st</sup> look analysis of these DNAs

⊗ Ordered 25 µg of  
pBR322 from Promega



Unfortunately it appears  
that none of the inserts are  
large enough to be the  
correct insert - will try  
inserting into pBR322 BamHI  
site

NB IDEA - Clone the problem

Will proceed with two approaches

① Using BamHI linkers, put the Hha/Xba fragment into pBR322 and  
will then transform a variety of hosts (HB101, SURE, ABLE, K-)

② Add RSV sequences downstream and upstream of the Hha/Xba  
insert in order to 'hide' the problem region

⊗ Called Greg Duke about recombination def. bacterial strain

To Page No. \_\_\_\_\_

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*Suzanne W. Kelle*

Recorded by

*David K. Clarke*

000313

From Page No.\_\_\_\_

Called Stratagene about yeast cloning system - Options ① yeast with centromere plasmids

② Non K-12 Strain of

E Coli (ATCC

Carried out TCID<sub>50</sub> on B93.2 V<sub>2</sub> DC5 V<sub>2</sub> 37°C/32°C

D.1/Unit 1

③ Use Baculovirus

Will read next mon/tues.

Spun down 1ml x 2 of Nucleocapsid prep - One tube contained about 200  $\mu$ M

of the 'old' nucleosynid prep. - Dried pellets - will set up 2 RT/PCR RNS from each pellet.

Prepared fresh 1.85M 2-Mercaptoethanol

Ordered Xho → Bst primer - will try to do 4.48kb RT/PCR RAN

5' - TGA C C C G A G C G C T T G A T A - 3'

Set up Kinasing of BamHI linker oligo ( $10 \mu\text{g}$ )  $\sim 1.25 \text{ hr}$  at  $37^\circ\text{C}$

10  $\mu$ L (10  $\mu$ g) of primer

$$5 \times 10$$

Sub Spermidine 15mm

ATP (10mm)

- ipl. Enz

H<sub>2</sub>O to 50% ... 1.25 hrs. at 37°C

Also set up RT/PCR rxns - Spun down ~ 1ml of ETOH ppt - dried  
resuspended in 4  $\mu$ l  $H_2O$  / 4  $\mu$ l H<sub>2</sub>O  $\Rightarrow$  Xca primer - Heated at 94°C for 2mins - snap cooled  
added ~ 1  $\mu$ l of Methylmer, 3mins RT, then ~ 1  $\mu$ l of  $\beta$ -mercaptoethanol, 3mins RT, then dispensed  
into PCR tubes and added 1  $\mu$ l of RTase  $\rightarrow$  1.25 hrs at 42°C - Proceeded to set  
up PCR rxns.

Set up on Xcy/Bum ④ and 3K sure

To Page No. \_\_\_\_\_

**Witnessed & Understood by me,**

Date \_\_\_\_\_

**Invented by.**

**Date**

George W. Kibbe

Recorded by

orded by David K Clarke.

000314



TITLE

Analysis of RT/PCR products / Preparation of Bam HI linker

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

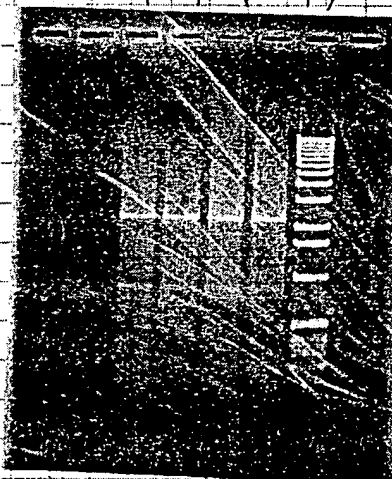
75

From Page No. \_\_\_\_\_

prepared 250ml broth for  $\phi$ X plasmid prepprepared 1% agarose gel for analysis of  $\phi$ X RT/PCR productsloaded 10 $\mu$ l of each RXN mixture

Chen from extracted RT/PCR rxns and EtOH pptd with a little glycogen

\* Ordered New  
RT/PCR Kit  
(Perkin Elmer)

~ 15 $\mu$ g/band in 1/4 X RT-PCR productsTotal of 300 $\mu$ g for 'New' and for 'Old'

Good - it looks as though the  
quality of the New RNA is also  
v. good

Will Digest the 'Old' product  
with Hha I and ~~Bst~~ Bst 1107 I  
for subsequent ligation the  
the 3.6K DNA and the Xba/Bam  
fragment

Annealed: 2 $\mu$ g of Kinased Bam HI linker to 2 $\mu$ g  
of unkinased Bam HI linker ① In NTE

Heated briefly at 90°C

then put at 37°C for 30mins

Then " " 32°C for 30mins

Then " " 27°C for 20mins

then " " 22°C for 20mins

allow cooling  
With 5min intervals between each annealing temp to

Set up own 250ml broth cultures

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suz W. Kbb

Recorded by

David K Clarke

0000315

From Page No. \_\_\_\_\_

There is no HhaI enzyme in the freezer (or HinPII - an isoschizomer of HhaI) - ordered 2,000 of each

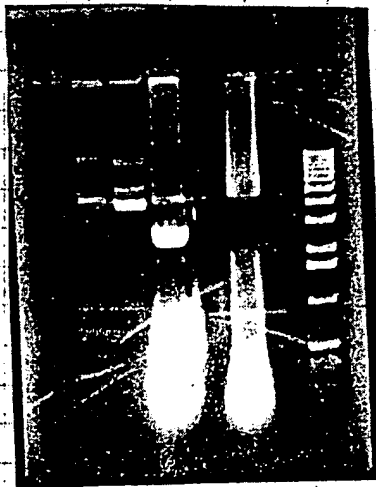
Spin down Bst 1107 I digested Hha/Xba RT/PCR DNA and stored dried at -20°C until ready for digestion with HinPII.

Meanwhile run a gel of the remaining Hha/Xba RT/PCR product

Electroeluted the DNA and EtOH ppt<sup>d</sup> with glycogen.

Also carried out plasmid preparations of Xba/Bam (2.6K) and 3K sure using the Qiaagen columns - Run out Spl (of ~600) on an agarose gel.

3K sure  
Xba/Bam 2.6K  
can plasmid  
Hha/Xba DNA (PCR)  
1Kb ladder



Will digest the

3.6K clone with HhaI or

~~Bst~~ HinPII and the

KpnI and XhoI (to find out orientation in the MCS).

Will digest the Xba/Bam clone with Bst 1107 I and Bam HI

It looks as though the

3K sure and Xba/Bam DNAs are okay and ~ the correct size.

Will know better following digestion.

3K SURE  $\approx 100 \mu\text{g Spl}^{-1}$  (Net 120  $\mu\text{g}$ )  
Xba/Bam  $\approx 2 \mu\text{g Spl}^{-1}$  (Net 120  $\mu\text{g}$ )

Can then reclone 3.6K/Hha-Xba/Xba-Bam frag back into pCR II vector  $\rightarrow$  Sure  
 $\rightarrow$  One shot etc.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Invented by

Date

Ang W. Klob

Recorded by

David K. Clarke

TITLE Analysis of purified Hha/Xba / Prep of pBR322 as Vector.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

77

From Page No. \_\_\_\_\_

Set up BamHI digestion of pBR322 - which cuts in the Tet<sup>R</sup> gene

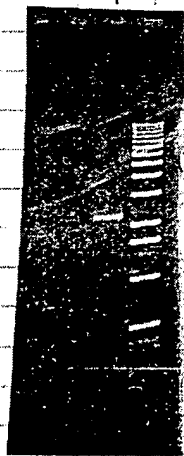
50µl final vol / 2µl Enz / 2µg pBR322 / 2hrs 37°C /

Then Phenol chloroform extracted and EtOH pptd prior to S.A.P.  
(Shrimp Alkaline phosphatase treatment) 5' phosphorylating ends.

Also spun down gel purified Hha/Xba PCR product - resuspended in

20µl H<sub>2</sub>O and analysed 2µl of this on a 1% Agarose gel.

H/Xba (purified)  
1Kb ladder



Good - there appears to be 50-bony of  
purified Hha/Xba RT/PCR product in  
2µl of the stock, giving a total of  
~ 500ng - This material will be  
used for the addition of BamHI  
linkers.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sign W. K. K.*

Recorded by

*David K. Clarke*

0000317

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE \_\_\_\_\_

Infection of Heps for more N capsid  
S.A.P. of Bam cut pBR322 + gel purification~~and ligation of Hha/Xba ( Bam ) to Bam linker~~

From Page No. \_\_\_\_\_

(checked) Hep-2 cells - they are ~ 70-80% confluent - will probably be  
okay to infect later this morning

Also did preliminary check on TCID<sub>50</sub> - again it seems that 32°C is the  
best temp for growth of RSV - this time would give a higher titer than

did. Will do a final Reading tomorrow.

10-30am Infected 10 x TCID<sub>50</sub>s, each with 1ml of V<sub>3</sub>H, 32°C

Set up SAP RNM on pBR322 (2µg) in 50µl - with ~2µl of

SAP 30min at 37°C - added another 1µl SAP + 30min more incubation

at 37°C - then added EDTA to 10mM and heat inactivated at 65°C for

15min. Meanwhile prepared 1% Agarose gel for purification of SAPs pBR322

Will set up ligation of 4µl (100ng) of purified Hha/Xba DNA with BamHI linker,

but first need to Kinase the Hha/Xba DNA - will do all today. Kinase reaction  
was as previously described in 50µl final vol; Due to recessed 5' end included a

1/2 hr incubation (following 70°C heat/shock cool (to denature the ends a little) to improve

the efficiency of Kinasing; then phenol chloroform extracted and EtOH ppt<sup>ed</sup> - will

set up the 2nd ligation with linker tomorrow.

Meanwhile completed electrolution of pBR322 (Bam cut - phosphorylated)  
and etOH ppt<sup>ed</sup> following phenol chloroform extraction

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Prof. W. K. Klotz*

Recorded by

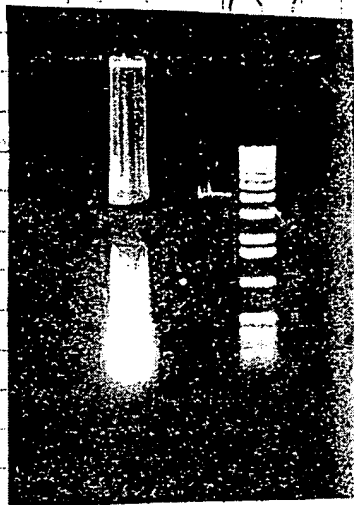
*David K. Klotz*



From Page No. 79

Meanwhile carried out (gel) purification of  $Hin$  P.I. /  $Bst$  H07.I cut  $\frac{Hhe}{Xca}$  RT/PCR

Eluted DNA from  
NA-45 paper - Phenol  
extracted and EtOH  
ppt<sup>ed</sup> with 10  $\mu$ l of stock glycogen



It seems that Promega have  
short changed us in pBR322 by  
a factor of 10. It appears there is  
only ~10ng of DNA in 1  $\mu$ l of  
purified, stock, phosphorylated,  
and  $Bam$  H.I. cut. (Total ~20ng.)  
However I should have enough DNA  
for this experiment.

Also set up 2<sup>nd</sup> digestion on 3.6K,  $Xca/Bam$ , and in 20  $\mu$ l final vol to  
facilitate gel purifcat.  
Split 3.6K DNA into 2 (4  $\mu$ g x 2)

A)  $Xho$  I cut (with  $Hin$  P.I. cut already  
done)

B)  $Kpn$  I cut (with  $Hin$  P.I. cut already  
done)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kibbe*

Recorded by

*David K Clarke*

TITLE Nucleocapsid Harvest / B9322 Infections / Digestion of Hha-Xba linker with Bam HI

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

81

From Page No. \_\_\_\_\_

Infected Vero cells with B9322 DI  $V_6DS_5V_2$  }   
 undil.   
 undil.   
 undil.

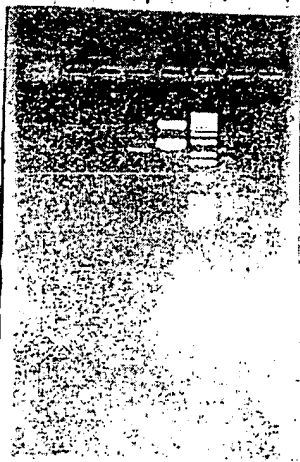
Absorbed ~ 3hrs at 37° before feeding and putting at 32°.

Began Nucleocapsid Harvest - as before - c.p.e. looked very good on the 10 TC150s.

Skipped second lysis buffer wash of pellet - went directly to T.E. wash.

Phenol chloroform extracted ligated Bam linker / Hha-Xba purified RI/PCR DNA and etoh. ppt'd with glycogen - Will set up Bam HI digestion in 30  $\mu$ l (~ 2hrs at 37° with 1  $\mu$ l (20 u) of enzyme; tomorrow

*Handwritten notes:*  
 - Hha/Xba linker  
 - B9322 check  
 - 1 kb ladder



The H/X frag. has been trimmed with Hha.P1 and Bst.110.7 I and gel purified  $\frac{1}{10}$  th loaded on gel.

It seems that the pBR322 stock is fine at 1  $\mu$ g  $\mu$ l<sup>-1</sup>

The recovery of H/X frag. is a little lower than expected (vs for pBR322) should probably make fresh NATS paper.

Put on nucleocapsid spin for O/N Run - 24K 5° 16hrs (2 gradients)

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Signature: S. W. K. K. K.*

Recorded by

*Signature: David K. Klathe*

000321

From Page No. \_\_\_\_\_

Prepared some fresh NA 45 paper for Electrolution of DNA from  
Agarose gels - stored at  $+4^{\circ}\text{C}$

Took off nucleocapid spin - harvested nucleocapid bands and collected  
by centrifugation after dilution in T.E buffer - Then resuspended in 500  $\mu\text{L}$  TE/pellet  
and set up SDS (0.2%) and proteinase K digestion.

Set up Bam HI Digestion on linker and gel purified Hha/Xba DNA (100ng)

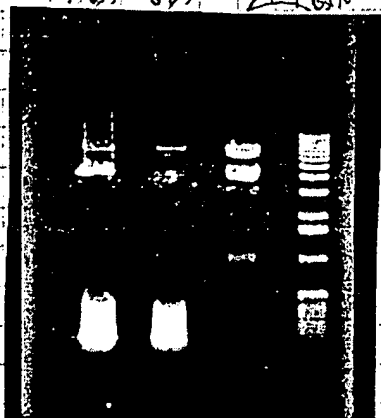
2hrs at  $37^{\circ}\text{C}$  -  $\mu\text{L}$  Enzyme - Phenol chloroform extracted and EtOH ppt<sup>ed</sup>

With glycogen - Will ligate this DNA with Bam HI cut/phosphatized pBR322

Will use <sup>50</sup>100ng ( $\frac{1}{4}$ ) of pBR322 prep and  $\frac{1}{4}$  (<sup>25</sup>100ng) of cut-linkered H/X fragment

Will do <sup>50</sup>200ng of pBR322 alone as con Will set up ligations tomorrow

May need to recut linker DNA due to high number of Bam HI  
sites generated by large amount of linker



KpnI cut is probably  
correct since it is the  
smaller DNA - will  
know better when the  
gel is rerun to check  
quantification

Xba/Bam Digestion appears  
to have an Xba 1Kb frag! but it  
looks as though it is at sub-mole  
gr ratio - perhaps there is some  
'Star' activity

Electroeluted all 3 DNAs - and  
phenol extracted/EtOH ppt<sup>ed</sup> with  
glycogen

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suz W. Koble

Recorded by

Daniel K. Clarke



Interviewing of Potential RAs.  
 TITLE B9320 Harvests / Quantitation of purified 3.6K and 2.6K clone DNA.  
 Redigestion of Bam linked Hha/Xba DNA.

Project No. \_\_\_\_\_

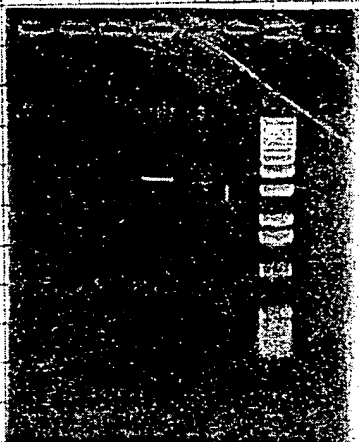
Book No. \_\_\_\_\_

83

From Page No. \_\_\_\_\_

Redigested Bam linked H/X DNA with new Bam (to counter xba linker)  
 Used 3 $\mu$ l of Bam in 6 $\mu$ l for 3hr at 37 $^{\circ}$ C. - extracted and ppt'd with glycogen  
 5 $\mu$ l H<sub>2</sub>O 3 $\mu$ l Xba 2 $\mu$ l Bam 1 $\mu$ l buffer

The electrophoresed products are in  
 low abundance - about 50-100  
 total in each - will need to  
 check protocol to find out what  
 is going wrong.  
 However - should have  
 enough for 1 experiment.



usually the slightly smaller  
 3.6K (KpnI) fragment is  
 the correct fragment

Will phosphorylate both fragments  
 tomorrow, and subsequently  
 ligate with ~10-20ng of  
 Hha/Xba DNA, then ligate  
 those products into KpnI/BamHI  
 cut vector

Harvested B9320 infections - Now showing  
 good c.p.e. in all cases

10<sup>-1</sup> V<sub>6</sub> DC S<sub>1</sub> V<sub>3</sub> 32 $^{\circ}$ C - D.I  
 Untd " 32 $^{\circ}$ C - D.I  
 refers to infecting virus (from previous harvest)

Eco cut empty vector - ligate -  
 result with Bam and KpnI -  
 ligate in frag. and transform  
 100ng. insert: 50ng vector.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Lyse W. Khlh*

Recorded by

*David K Clarke*

000323

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE \_\_\_\_\_

Eco Digestion of PCR II vector  
 Analysis of Bam cut, Bam linked H/x DNA.  
 DNA ligation of linked Bam cut H/x DNA to Bam cut phosphorylated

pBR322

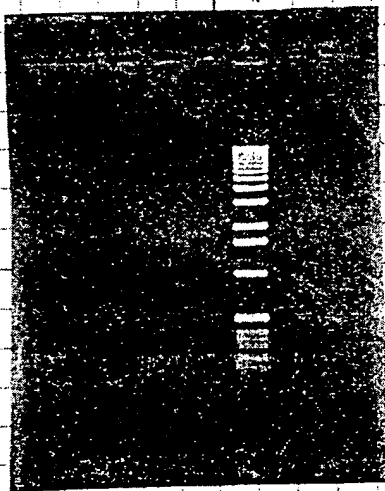
From Page No. \_\_\_\_\_

Prepared L-Agar for tomorrow's transformations — poured Amp<sup>r</sup> plates.

Prepared 1% Agarose gel for analysis of Bam cut linked H/x DNA — will analyse 1/4 of total (resuspended in 120  $\mu$ l)

Handy 120  $\mu$ l buffer

It may be necessary to kinase the PCR primers to give a higher recovery of DNA (by eliminating this step from the procedure)



There is no obvious band of H/x (2.5K) DNA visible — decided to destain the gel a little to see if any band became more obvious.

(see gel below)

The red arrow below, points to the merest whisp of a band of DNA at the 2.5K position

Set up EcoRI digestion on PCR II TA vector —  $\phi$  extracted and EtOH ppt<sup>d</sup>



Went ahead with DNA ligation

Used 2  $\mu$ l Vector (2.20-3.25  $\mu$ g)

→ All remaining H/x linked DNA (4  $\mu$ l)

heated at 65°C 15 min in 10  $\mu$ l buffer

for 30 min to 1  $\mu$ l Enz and 11.0  $\mu$ l 15°C

left at R Temp 90°C, along with

an equivalent pBR322 only control

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Serge W. Kibuk

Recorded by \_\_\_\_\_

David K Clarke

Esty/Xho RT/PCR  
Religation of Eco cut PCR.  
Phosphatase 3.6 KpnI and 2.6K DNA fragments  
Transformation with pSR22+ linked H/x DNA.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

85

From Page No. \_\_\_\_\_

Set up phosphatase rxn on 3.6K (KpnI) DNA and 2.6K DNA (The low recovery level is probably due to insolubility of DNA after elution from NA45 paper due to the presence of gel agar) - Did 2x (15mins 37°C / 15mins 56°C) each time with 1 µl of phosphatase (SAP) - Heat inactivated at 65°C / 10mins EDTA for 15mins, then phenol chloroform extracted and ppted

Set up religation of EcoRI cut PCR - 2hrs at 37°C in 20µl 1st rxn then phenol extracted and ppted in preparation for KpnI/Bam double digest (will save 2µl for gel analysis following religation)

Spin down 1/2 of each RNA prep<sup>W/ cap</sup> and proceeded to set up RT/PCR RXNS as before

Set up bacterial transformations - using Sph/ transformation of SV ligations between pSR222 and Bam linked H/x DNA.

Used 'SURE' cells  
'ARLEK' cells  
'DH5α' cells - (used as Gen. also)

plated out 500µl from each on a separate large petri dish.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Steve W. KVL

Recorded by

Daniel K Clarke.

000325

From Page No. \_\_\_\_\_

Checked bacterial transformants - There were ~100 on the large control plate, indicating that the phosphorylation was not complete - However it was

## Galloway Lab Strain Collection

05/02/1994

Page

1

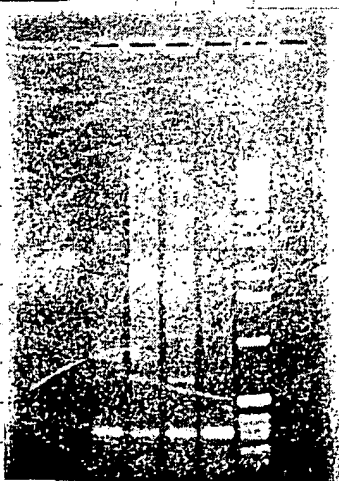
Location 232  
 Name JC9387  
 Aka S741 IN G. Smith's lab.  
 Total\_size 0.000  
 Host\_cell Host cell  
 Vector  
 Size\_kb 0.000  
 Cut\_with  
 Insert  
 Ins\_size 0.000  
 Origin  
 Release\_by  
 Resistance

Comments Used by Dan L. to maintain the HSV ori-bearing plasmids. AB1157 background [thr1, leu6, thi1, lacY1, galK2, ara14, xy15, proA2, his4, argE3, str31, lxx33, mtl1, met-(weakly), su-] and recB21, recC22, sbcB15.

References Construction of strain described in J.R. Gillen, Ph.D. thesis, UC Berkeley, 1974. Lockshon and Galloway J. Virol. 58, 513 (1986)

Set up Bam HI digestion  
 of religated EcoRI cut  
 PCR II Vector - 2 hrs at 37°C  
 in 50 µl with 1 µl Enz

Set up ligation of  
 H/x and x/H, used  
 25 µg of each in 50 µl  
 with 1 µl Klenow v/hr at  
 37°C



Gel looks discouraging - do not see  
 the appropriate 4.5 kb RT/PCR product  
 expected

Do not see religated PCR II  
 DNA either - however will proceed  
 with Bam and Kpn I digestion of this  
 material

Set up 96 mini preps of  
 JC9387 (from Galloway Lab)  
 in plain L-B broth - no  
 antibiotics.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kline*

Recorded by

*David K. Clarke*

H/Xho and H/Xca RT/PCR RXNS.  
TITLE: KpnI digestion of pBR322 (Sam Cut)

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

87

From Page No. \_\_\_\_\_

Set up KpnI digestion on Bam HI cut pBR322 - Phenol extracted  
and EtOH pptd.

Set up RT/PCR Hha/Xho and (H/Xca x4) - Used 3x1ul of EtOH ppt  
(x1)  
Used newest RT/PCR kit  
Used Kinased oligos for Hha/Xca RT/PCR Not Kinased for Hha/Xho.

Streaked out JC9387 bacteria on an L-B Agar plate; also freeze  
down 2x1ul ampoules in 15% glycerol as long term stock.

Ordered some vector from Invitrogen pCDNA3.1

Prepared 0.1M CaCl<sub>2</sub> (50ml) - will autoclave tomorrow

Will redo pBR322 cloning.

- ① Use Kinased oligos for RT/PCR (as above)
- ② Add Kinased linker and ligate (x1ul x3)
- ③ Gel purify linked DNA
- ④ Trim with Bam HI
- ⑤ Ligate into phosphorylated pBR322.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Steve W. Kline*

Recorded by

*David K. Clarke*

000327

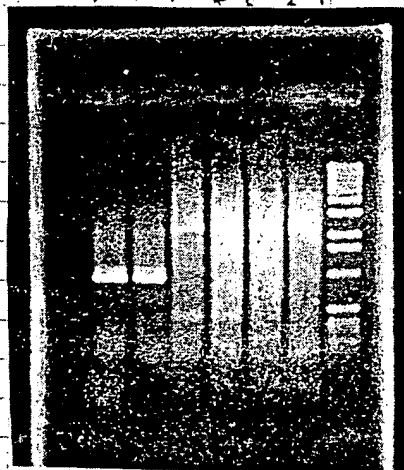
From Page No. \_\_\_\_\_

Autoclaved 0.1 M  $\text{CaCl}_2$  in preparation for Camp cell production.

prepared 2 x 1% Agarose gels - one for analysis of RT/PCR products

are for analysis of the components going into the

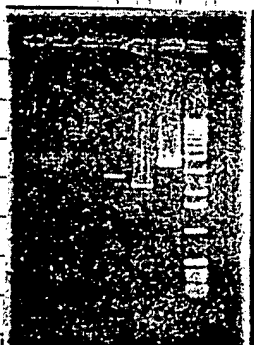
11h/xen  
K<sup>+</sup>  
11h/xen  
+ 2 1)



The 11h/xen products are not as plentiful as I would have liked - will repeat with 1ul of EtOH ppt and kinase against tomorrow.  
Meanwhile Clonform extracted and EtOH ppted with a little glyc. Will utilize only ① and ④.  
The 11h/xen products look fine.

loaded 1% Agarose gel with 2.6K, 3.6K, 11h/xen, along with Bam/HpaI cut pCII.

2.6K  
3.6K  
11h/xen  
+ 1ul



Set up TCID<sub>50</sub>s on B9320

10<sup>1</sup> V<sub>6</sub> DCS, V<sub>3</sub> < 10<sup>1</sup> untl  
untl V<sub>6</sub> DCS, V<sub>3</sub> < 10<sup>1</sup> untl

Will use 1ul of 3.6K/Spl of 2.6K/ and  
2ul of 11h/xen and 100ng of pCII  
Vector.

Witnessed &amp; Understood by me,

Date

Invented by

Date

To Page No. \_\_\_\_\_

Steve W. Chole

Recorded by

David K. Clarke

000328

Get purification of Hha/Xho frag.

Project No. \_\_\_\_\_

TITLE: RT/PCR Rxn using Kinased Hha/Xca digests.

Book No. \_\_\_\_\_

89

From Page No. \_\_\_\_\_

prepared 1% Agarose gel and run out Hha/Xho RT/PCR  
DNA; for NA45 paper purification. Eluted DNA - phenol extracted  
and EtOH ppt<sup>d</sup>

Set up RT/PCR Reaction (on 1ul of EtOH ppt ~~go~~ containing genomic  
RNA) using Kinased Hha/Xca primers.

Revised protocol for 3.6K, 2.6K, Hha/Xca and pcII plasmid cloning.

- ① Digest Clones appropriately to give ends of DNA, phosphorylate  
appropriately and then gel purify fragments
- ② Double Digest pcII DNA - gel purify
- ③ Do tetrapartite ligation and transformation.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Steve W. Kibbe*

Recorded by

*David K. Clarke*

0000329

G. American  
408 988-1776

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Gel analysis of RT/PCR products  
Digestion of pcII DNA vector with Bam/KpnI.

From Page No. \_\_\_\_\_

Prepared L-broth for growth of competent cells; prepared 400 ml ± Agar  
for Amp<sup>+</sup> plates. -

Prepared 1% Agarose gel for analysis of Hha/Xho RT/PCR products and  
purified Hha/Xho DNA.

Hha/Xho  
Hha/Xho  
Hha/Xho  
Hha/Xho

Interesting to see  
variation from Run to  
Run II

Will gel purify all 4 (2)  
H/X K<sup>+</sup> DNA tomorrow



set up BamHI digestion  
of pcII DNA - let run  
~ 2hrs at 37°C in 50µl  
final vol, with 2µl shock enz.  
Then phenol/chloroform extracted

Set up o/n ligation between pcII vector and Hha/XhoI DNA

Used 2µl Vector  
1µl DNA  
2µl x 2  
14µl H<sub>2</sub>O  
1µl Enz  
20

left o/n at 12°C

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Steve W. Kibbe

Recorded by

David K Clarke

000330



TITLE

Get purification of Hha/Xba K<sup>+</sup> DNA  
 Ligation of Kinased linkers to Hha/Xba DNA  
 Get purification of Hha/Xba K<sup>+</sup> DNA

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

91

From Page No. \_\_\_\_\_

Transformation of 'SURE' and T/A cells with Hha/Xba DNA  
 KpnI digestion of Bam Cut pcDNA II

Prepared 1% Agarose gel for purification of K<sup>+</sup> Hha/Xba RT/PCR products.

Meanwhile set up KpnI digestion of Bam Cut pcDNA II - in 50  $\mu$ l  
 37°C for ~2 hrs 2  $\mu$ l Enz - Then freeze away prior to gel purification (tomorrow)

Set up 3 transformations Hha/Xba in 'SURE' cells  
 Hha/Xba in T/A cells  
 Xho/Xba in 'SURE' cells

Linker is at 100 ng  $\mu$ l<sup>-1</sup> in H<sub>2</sub>O ~ 10 nt in length which is ~ 200 x smaller

than Hha/Xba(K<sup>+</sup>) - so ~~up to 200 ng of Hha/Xba~~ would require ~~200~~ of linker

Will shoot for a ~~500 ng of linker~~ but will wait until I get a sufficient quantity of pure Kinased Hha/Xba DNA before proceeding - may need

to adjust the RT/PCR conditions for the Kinased oligos. i.e.

① Raise the annealing temp

② Alter the <sup>rel.</sup> amount of oligo going into the rxn

Will check DNA after linker ligation

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Invented by

Date

Suzanne W. Kelle

Recorded by

David K. Clarke

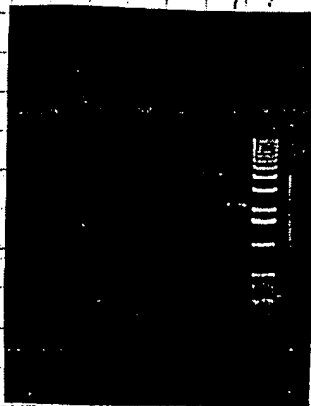
0000331

From Page No. \_\_\_\_\_

Checked bacterial transformants - there are numerous clones on all plates.  
However, <sup>all</sup> many of the clones on the TA Hha/xho plates had a  
slightly blue colour - streaked out the palest blue colour colonies on a  
master plate; also streaked out 'Hha/xho SURE' colonies and 'Xho/Xba  
SURE' colonies, on a master plate - will set up ON cultures tomorrow.

Prepared a 1% Agarose gel for gel purification of Bam/KpnI int. pcII DNA.

Set up ON J6 <sup>9387</sup><sub>3879</sub>



It looks as though there is ~10ng  
of DNA in 1  $\mu$ l = 100ng total  
remaining. Will ligate a (mol)  
5x excess of Bam linker to  
this DNA on at 12°C (will  
check 1/10 in a gel)

This will be ~5ng of  
Linker (use 1  $\mu$ l of 1/20 dil<sup>n</sup> of stock)

Must check mp of linker and  
ligate ~5°C below that (if  
consistent with a 12°C lig<sup>n</sup> temp)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Invented by

Date

Serge W. Khele

Recorded by

Daniel K Clarke

Preparation of Competent JC 9387 cells.  
TITLE o/n ligation of linker and H/X K<sup>+</sup> RI/RRE DNA

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

93

From Page No. \_\_\_\_\_

Prepared a 1% Agarose gel for analysis of gel purified  
Bam/Kpn (1  $\mu$ l from 1  $\mu$ l)

Inoculated 200ml L-b (prewarmed) with 1ml of o/n JC 9387  
pcII DNA - Bam/Kpn cut - gel purified  
1Kb ladder.

Competent cell prep<sup>n</sup>

NB All solutions and centrifuges etc  
should be chilled 0°-4°C

① Inoc prewarmed L-b (200ml) with 1ml  
of o/n culture.

② Grow with vigorous shaking until  
 $A_{600} \approx 0.3 - 0.5$

③ Chill on ice - then spin down at  
3K r.p.m. for 3-4 mins.

④ Decant super and resuspend cells in  $\frac{1}{10}$  vol 0.1M  $CaCl_2$  (chilled)

⑤ Let sit on ice for 30 mins

⑥ Respin at 3K r.p.m. for 3-4 mins

⑦ Decant super and resuspend at  $\frac{1}{10}$  orig. vol. in 0.1M  $CaCl_2$

⑧ Leave on ice 1hr  $\rightarrow$  o/n and/or freeze in EtOH/dry ice in 15% Glycerol

pcII DNA looks good at 2.9Kb

There appears to be ~ 50-100ng in

1  $\mu$ l - leaving 500-1000ng total  
remaining

Will set up 4 way ligation

later in the week when I have  
competent JC 9387 tested for  
competence

To Page No. 94

Witnessed & Understood by me,

Date

Invented by

Date

*Suzanne W. Kille*

Recorded by

*David K. Clarke*

0000333

From Page No. 93

Checked TCID<sub>50</sub> of B9320 - It is clear that at this point diluted virus does best during infection - either dilute a previous wash/infection stock to  $10^{-1}$  before infecting or used a wash stock from a previously dilute infection - See final TCID<sub>50</sub> Read tomorrow

Set up on cultures of 14 clones each from

Sure	Hha/Xho
Sure	Xho/Xca
T/A	Hha/Xho
+ 2 controls (T/A)	

Set up  $\lambda$  phage ligation between Kinased gel purified Hha/Xca DNA

Diluted stock primer  $1/20^{th}$  and used  $1\mu l$  (50ng) in a ligation mixture containing  $\sim 100ng$  of Hha/Xca (lig) in a final vol of  $20\mu l$

$2\mu l$  X10

$1\mu l$  buffer

$10\mu l$  H/X

$6\mu l$  H<sub>2</sub>O

$1\mu l$  enz.

$20\mu l$  on  $12^{\circ}C$ .

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Scarp W. Kibbe*

Recorded by

*David R. Clarke*

000334

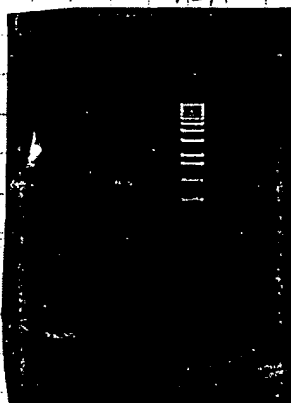
Trial Transformation of competent JC 9387  
Mini prep analysis of T/A and sure H/xho and xho/xca clones.

Project No. \_\_\_\_\_  
Book No. \_\_\_\_\_

95

From Page No. \_\_\_\_\_

Prepared 1% Agarose gel for analysis of o/n ligation products (linker +  
hybrid 1.1 KB ladder) Kinased H/xca



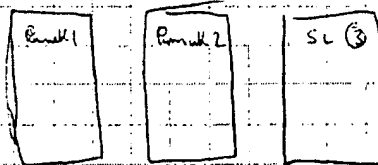
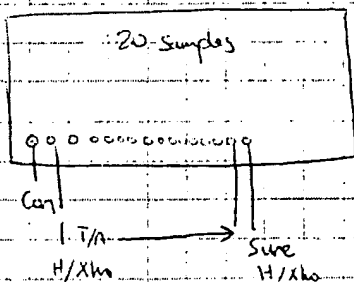
looks okay - there is still 5-10 kb in  
this sample (1% of total)

Phenol chloroform extracted and

EtoH ppt'd with 1pt of glycogen

Went ahead with analysis of 44 mini preps - 14 from each of the 3 transformation  
groups 'Sure' H/xho; 'Sure' xho/xca; T/A H/xho

Ran out ccc DNA on x gels using pCII (2.9 kb) DNA as control - will be  
looking for ~ a 2 kb difference for H/xho and 2.3 kb diff. for xho/xca



Phenol chloroform extracted and EtoH ppt'd  
linked K1 H/xca DNA

To Page No. 96

Witnessed & Understood by me:

Date

Invented by

Date

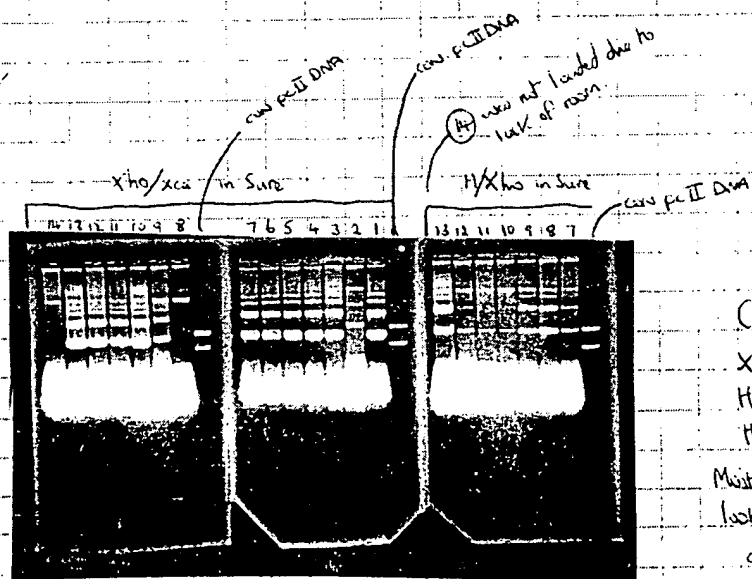
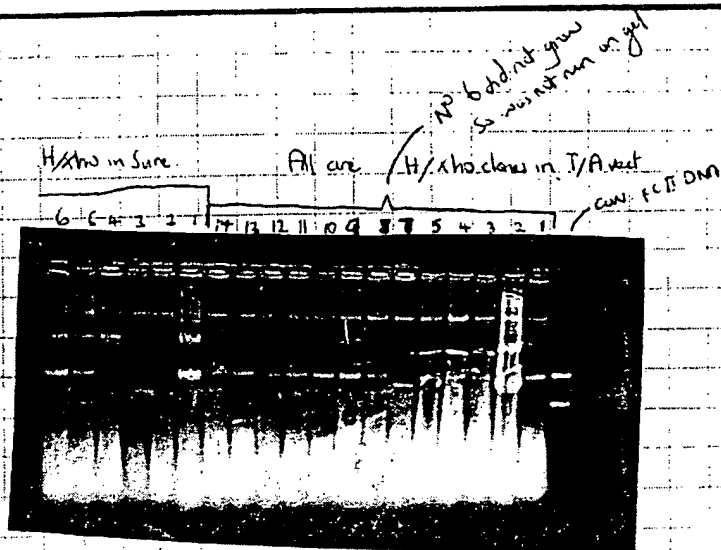
Sup. W. Kibbe

Recorded by

David K. Cluthe

0000335

From Page No. 13



Clone No 2 in 'Sure'  
Xho/xca looks as though it could be the one that I need.  
Most of the H<sub>ho</sub>/xho clones look good in both T/A and 'Sure' hosts.

The Xho/xca insert should be ~ 1,358, vs 976 for H<sub>ho</sub>/xho  
(This fits with the subtle difference in size of the ccc molecules)

Will digest 10µl of ② with EcoRI + PvuII and 9 + 8 with XhoI  
T/A 'Sure' and H<sub>ho</sub>  
2 hrs 37°C

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Steve W. Kille

Date

Invented by

Date

recorded by

David K. Clarke

From Page No. \_\_\_\_\_

Checked transformation efficiency with pc II DNA in JC 9387. - Looks very good (200 pg gave v. many colonies)

$$22 \text{ colonies/pg} = 2.2 \times 10^6/\text{pg}$$

Phenol chloroform extracted ② Xho/Xba in 'Sure'

④ Hha/Xho in T/A

⑧ Hha/Xho in 'Sure' and EtOH pptd prior to

double digestion ② with Xho and Bst 110.7 I.

④ + ⑧ Xho and Hha

Did Xho digestions 1st

Also set up Bam HI digestion on linked H/Xba DNA (K<sup>+</sup>) - with a

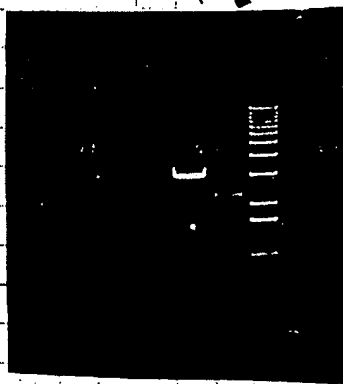
Bam control (pc II DNA) Run 1% Agarose gel for analysis

Will infect TC 75s with "10<sup>-1</sup> V<sub>6</sub> DCS, V<sub>3</sub> - Until" at 10<sup>-1</sup> ①

"Until V<sub>6</sub> DCS, V<sub>3</sub> - 10<sup>-1</sup>" Until ②

Used 0.75ml for each infection. and adsorbed for ~ 3hrs.

Bam cut all con.



Bam HI cut K<sup>+</sup> linked Hha/Xba DNA (1/5 of total) ~ 40ng remaining.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

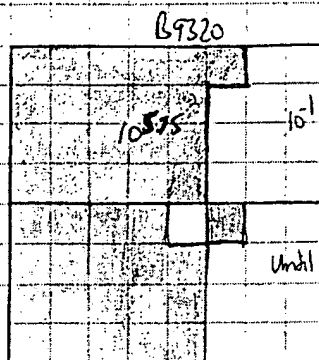
Date

*Sgt. W. Kibler*

Recorded by

*David K. Clarke*

From Page No. \_\_\_\_\_

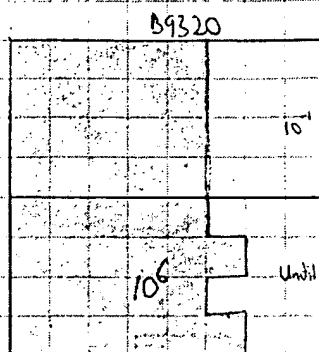
"Unit V<sub>6</sub> DCS, V<sub>3</sub>"

Looks as though a general

pattern is emerging - best to

infect cells with dilute (10<sup>-1</sup>) virus when coming from a high titer stock produced from an uninfected infection.

32

"10<sup>-1</sup> V<sub>6</sub> DCS, V<sub>3</sub>"OR with uninfected virus from a high titer stock produced from a dilute (10<sup>-1</sup>) infection.

Completed double digestion on mini prep DNAs and freeze away for gel analysis tomorrow.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne W. Krole

Recorded by

David K. Clarke

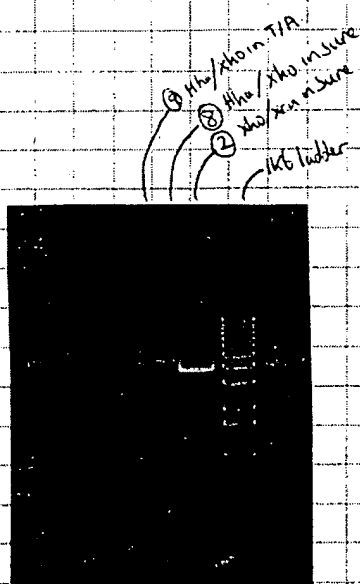


O/N ligation of 3.6, 2.6 H/xca int pE.DNA and H/xca into pBR322. Project No. \_\_\_\_\_  
 TITLE Gel analysis of Potential Xho/xca and Hha/xho clones Book No. \_\_\_\_\_

99

From Page No. \_\_\_\_\_

prepared L-Agar for tomorrow's transformations ~ 400ml  
 poured agar plates - dried and stored at 4°C  
 Prepared 1% Agarose gel and ran analysis of ② ⑧ ⑨ clones from  
 double digestion



Good it looks as though  
 all the clones contain the correct  
 inserts and the problem region(s)  
 have now been cloned

Set up O/N ligation

①	4 way	②	H/xca to pBR322
KpnI/HinPI	1µl 3.6K DNA (10:20ng)		2µl (10ng) H/xca - linked K <sup>+</sup> - Bam/Cut
Bam/Bst	5µl 2.6K DNA (10:20ng)		5µl (50ng) pBR322 - Bam/Cut - phosphorylated
HinPI/Bst	2µl H/xca DNA (5ng)		10µl H <sub>2</sub> O
	2µl pE.DNA (100ng)		2µl x10 buffer
	2µl x10 buffer		1µl Enz
	7µl H <sub>2</sub> O		20µl
	1µl Enz		

12°C O/N

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Sgt. W. Kibbe

Recorded by

David K Clarke

0000339

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

Transformation of 'SURE', JC9387, DH5 $\alpha$  with  
TITLE tha/xca/Vater DNAs - B9320 Harvest -

From Page No. \_\_\_\_\_

Checked c.p.e. on monolayers - not V. extensive - will leave harvest

until later in the afternoon

Carried out the following bacterial transformations

- 5  $\mu$ l (of 20  $\mu$ l) from each  
ligation mixture for  
each transformation.
- ① SURE cells with pII DNA ligated to R6K, 2.6K, A/xca DNA
  - ② DH5 $\alpha$  " " " " " " " "
  - ③ JC9387 " " " " " " " "
  - ④ SURE cells with pB322 ligated to Bam linked
  - ⑤ DH5 $\alpha$  " " " " " " " "
  - ⑥ JC9387 " " " " " " " "

Plated out 50  $\mu$ l from each transformation mixture into a large  
petri dish with Amp<sup>r</sup> L-AgarHarvested B9320 V<sub>6</sub> DCS, V<sub>4</sub> 10<sup>-1</sup> and V<sub>6</sub> DCS, V<sub>4</sub> Undil - Carried out  
TCID<sub>50</sub>s on both populations.

Interviewed Lisa Paganini

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Steve W. Kuhl

Recorded by

David K. Clarke

TITLE: Master plated potential Hha/Xba Clones

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

101

From Page No. \_\_\_\_\_

Checked bacterial transformation - there are too many to count on the pCIT DNA cells (Sure, DH5 $\alpha$ , Jc9387) - possibly due to incomplete cutting of one or other of KpnI or BamHI - Will repeat the transformation with 1 $\mu$ l of ligation mixture and dilute out the transformed cells to 1/1000 / 1/100 / 1/10 /

DH5 $\alpha$  and Sure with gave colonies with pBR322 (JC didn't - probably due to lower transformation efficiency) - Plated out thirty for each set on a master plate Amp<sup>r</sup> - will test on tetr<sup>r</sup> plates tomorrow. —

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sgt. W. Kihle*

Recorded by

*Daniel K. Kihle*

000341

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE Testing Tet<sup>R/S</sup> phenotype of potential H<sub>h</sub>/Xa clones

From Page No. \_\_\_\_\_

Prepared 200 µl L-Agar Tet<sup>R</sup> to streak out clones grown  
on an Amp<sup>R</sup> master plates; if they contain an insert then the tet<sup>R</sup>  
gene should be inactivated  $\equiv$  Tet<sup>S</sup>

Continued to work on invention disclosure form.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Steph W. Kibbe

recorded by

Daniel K. Clarke

Streaking out master plates with RV clones.

TITLE: On minus of potential Hhu/Xca clones.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

103

From Page No. \_\_\_\_\_

Prepared 500ml L-Agar (Amp<sup>r</sup>) for restreaking of clone banks - relevant clones only.

Will master plate the following clones.

① 3K clones (Virus 5' end)

A) NCTA3

B) NCTA4

C) NCTA5

D) ~~NE14~~ V NE14

Also 3K in 'SURE'

② 1.5K clone

A) clone #2 only - if new the only one identified in batch & Wmini preps checked.

③ 2.25K clone

A) 2

B) 4

C) 6

D) 7

④ 2.6K clone (also known as Xca/Bam)

A) 2

B) 4

C) 8

D) 9

⑤ 2.3K (Hhu/Xca)

To be fully figured out yet -  
So far it has been divided in two

Hhu/Xho + Xho/Xca

A) 1

B) 10

C) 8

D) 9

in 'SURE'

A) ② in 'SURE'

⑥ 3.6K (Virus 3' end)

A) 1

B) 3

C) 4

D) 7

Grown on at 37°C

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Suz W. Kibbe*

Date

Invented by

Date

Recorded by

*David K Clarke*

0000343

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE \_\_\_\_\_

From Page No. 103.

Set up 9/1 mini-preps on potential DH5 $\alpha$  pBR322 Hha/Ka clones  
(Those which were showing Tet<sup>S</sup> Amp<sup>R</sup>)

Witnessed &amp; Understood by me,

Date

Invented by

Date

To Page No. \_\_\_\_\_

*Sgt. W. Kelle*

Recorded by

*David K. Clarke*

000344

TITLE

Mini prep analysis of potential *thy/xca* clones in pBR322

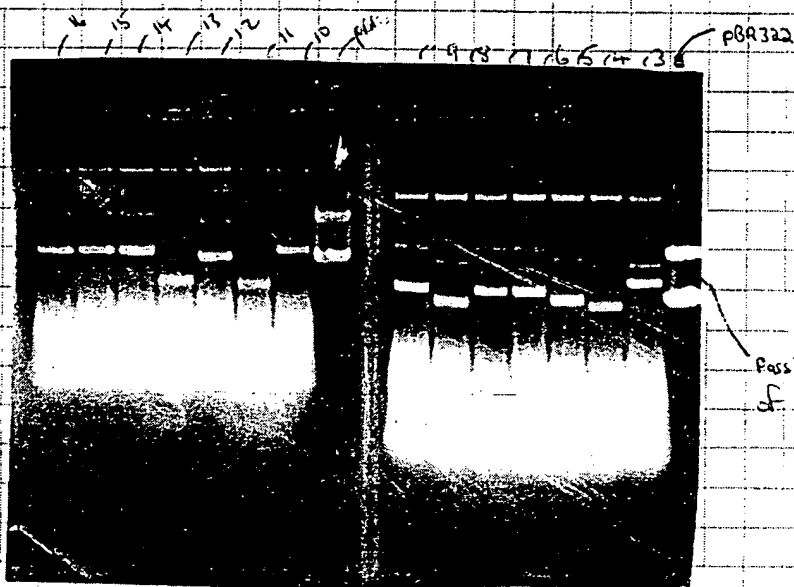
Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

105

From Page No. \_\_\_\_\_

Carried out mini prep analysis on potential *thy/xca* clones



Stored clone  
banks at 4°C  
(Restrained some of  
them)

It looks as though  
they have undergone  
various deletions and  
rearrangements  
  
Possible non-deleted form  
of clone

Set up o/n mini preps of

- Clone ① *thy/xho* in Sure
- ② *xho/xca* in Sure

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sage W. Kelle*

Recorded by

*David K. Clarke*

000345

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

O/N xho/xca and Hha/xho large scale preps.  
TITLE Method for Hha/xho and xho/xca link up.

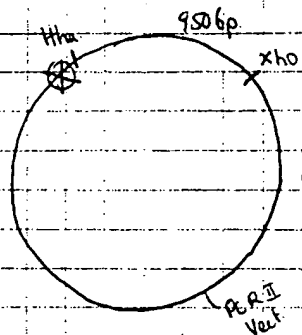
From Page No. \_\_\_\_\_

Put on cultures of xho/xca and Hha/xho on ice prior  
to setting up and large cultures later this evening

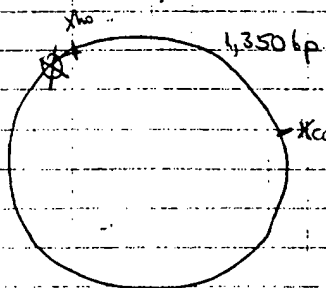
A) Clone 2 xho/xca

B) Clones 7, 8, 9, 10 Hha/xho

} restreaked on new master plates.



cut ~~Hha~~ ~~Hha~~ and  
xho cut

↓  
Purify fragment.may need to determine  
orientation first.

xho cut and gel purify. (Need to determine rel.  
position of xho I. site)

checked TCID<sub>50</sub> - Will make a final reading tomorrow

Set up on cultures of ② sure xho/xca and ⑧ sure Hha/xho

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serg W. Kurbak

Recorded by

David K. Clarke



**TITLE**

Plasmid preps  $hho/xho$  and  $xho/xcc$ .

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

107

**From Page No.\_\_\_\_**

10 5.5

10 6.2

B9320  
V<sub>6</sub>DC5, V<sub>+</sub> 32°C  
10<sup>-1</sup>

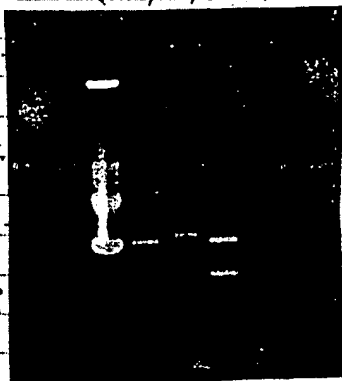
Will infect 6 x TC150 with  
Undil. virus from the 10<sup>5.5</sup> stock.

and  $6 \times 10^{15}$  with  $10^{-1}$  d  
of  $10^{6.25}$  stock

Set up bacterial transformation using DH5 $\alpha$ , SURE, and JC938.7 cells;  
Used  $\mu$ l of ligation mixture in each case - plated out  $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$   
(2  $\mu$ l of each) from each cell transformation mixture

Possible  
Chromosomal  
DNA

actually looks  
as though the  
putative chain did  
actually plasmid  
ma.



GOOD; both plasmids  $Xho/Xca$  (1) and  $Hha/Xba$  (8) appear to have been stably amplified.

**To Page No.\_\_\_\_\_**

**Witnessed & Understood by me,**

**Date**

**Invented by**

**Date**

Sgt W. Kimbrell

Recorded by

ordered by Dawn K. Clarke

000347

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Master plate of Potential 8.5K clones.

Restriction analysis of (2) and (8)

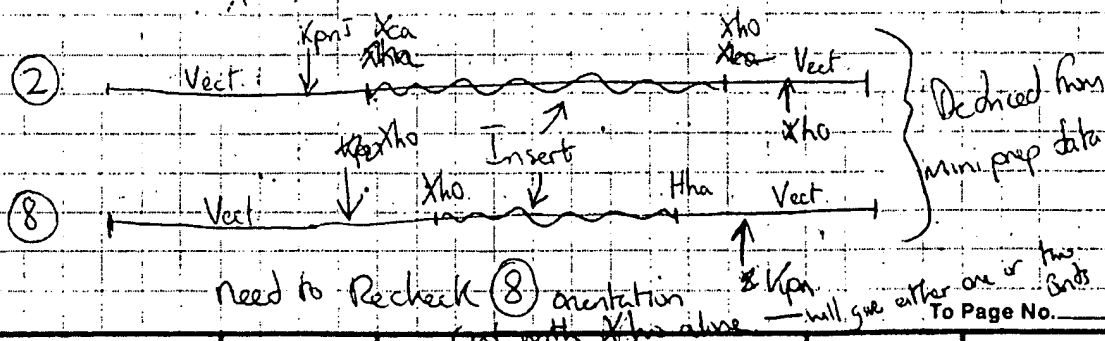
From Page No. \_\_\_\_\_

Checked bacterial transformations - streaked out 36 potential 8.5K clones from SURE, JC9387, DHSX transformations

Will digest 20 $\mu$ l from each plasmid prep with both KpnI and XhoI - this will determine the orientation for each of the clones (2) and (8).

Preval cloform <sup>extracted</sup> ppt<sup>ed</sup> and EtOH ppt<sup>ed</sup> between digestions

Prepared and ran a 1% Agarose gel for analysis



Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge W. Kibbe

Recorded by

Daniel K. Clarke

TITLE

Further restriction analysis of ② and ⑧

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

109

From Page No. \_\_\_\_\_

Took potential 8.5K clones from incubator and stored at 4°C

Set up the following digestions

② - Xho I only (to check orientation of clone in vector - will give either one or two bands)

⑧ Xho I only (to check orientation)

② Bst 1107 I (to verify presence of this site)

Spin down 40µl ② and 20µl ⑧ - set up digestions

Prepared a 1% Agarose gel for analysis

Xho 3 Xho 2 Bst 1107 I ②  
1K ladder

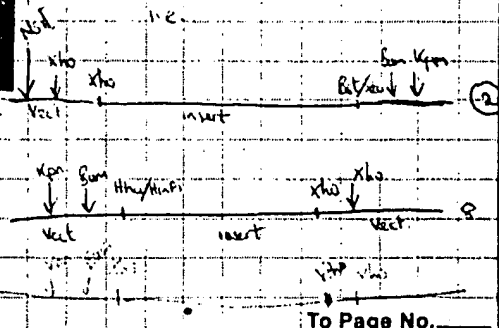
Will proceed by ~~Boa~~ <sup>EcoRI</sup> Xho  
cutting ②  
and ~~Boa~~ Xho cutting  
⑧



Everything looks fine

From gel can deduce that  
Xho I site of insert end and  
Xho I site of vector are on  
the same side

40 bp  
fragment from  
MCS Xho to CIR Xho  
site.



Witnessed & Understood by me,

Date

Invented by

Date

*Sege W. Kuhl*

Recorded by

*David K. Clarke*

To Page No. \_\_\_\_\_

000349

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

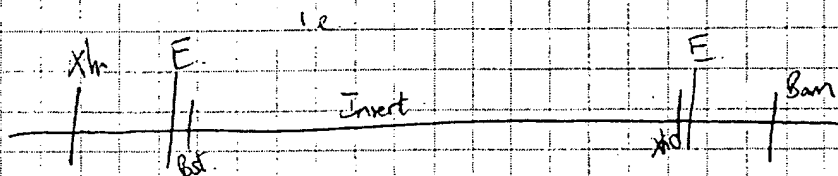
TITLE

Infection of Vero's for B9320 antigen.

Double digestion of (2) and (8) / O/N minus for 8.5K clones

From Page No. \_\_\_\_\_

Will spin down 200pl each of (2) and (8) for double digestion  
 Will have to flip the 'insert' of (2) around (within the EcoRI sites) to generate  
 the correct configuration within the M.C.S., to do this will digest ~ 500ng of  
 (2) then do religation and transformation (will check digests on a gel)



Will also ~~cut~~ (8) with Xho I (20µl)

Infected 12 x TC 50s with B9320 virus

6 Flasks with <sup>UNO2</sup>  $V_6$  DCS,  $V_4$  32°C diluted 10<sup>-1</sup> and 1.1ml used.

6 Flasks with 10<sup>-1</sup>  $V_6$  DCS,  $V_4$  32°C Undiluted and 1.0ml used/Flask

Adsorbed ~ 3hrs (to 3-45pm)

Meanwhile prepared a 1% Agarose gel for analysis of restriction digests.

Xho (8)

EcoRI (2)

Digestions look okay

Set up O/N minus  
for potential 8.5K clones

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sign. W. K. K. K.

recorded by

David K. Clarke

000350

Mini prep extraction and analysis for potential 8.5K clone  
TITLE O/N ligation of EcoRI cut (2) Project No. \_\_\_\_\_  
Book No. \_\_\_\_\_

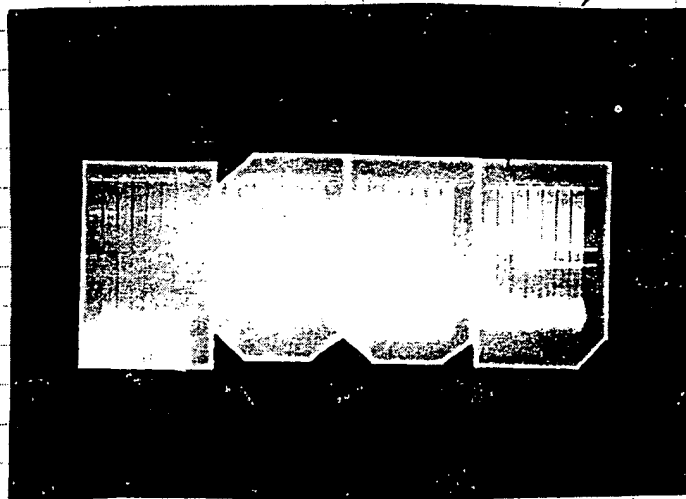
111

From Page No. \_\_\_\_\_

Carried out 30 mini prep DNA extractions on potential 'SURE' and 'JC' clones which might contain the 8.5K clone

Prepared 4 x 1% Agarose gels and ran out samples with ecc pc II DNA as control marker

Could try to do the same procedure in 3 steps using the Hha/Xho and Xho/Xba fragments



Only clone N°6 from the 'SURE' batch

Contained an insert, but it does not appear to be large enough to contain the

3.6  
2.3  
2.6

8.5Kb clone

however - should see what size the insert is - it

it maybe cut with the 2.6Kb ~~But not~~ N°6.I

Set up O/N ligation of Eco cut (2) (for religation) at R Temp in 24hr with 1ul enzyme

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Steve W. Kuhl*

Recorded by

*David R. Clarke*

0000351

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Gel purification of Xho cut ⑧

Transformation of sure cells with religated Eco cut ②

~~Rest of digestion of SURE ⑥ - SSK~~

Nsi I

From Page No. \_\_\_\_\_

Checked cpe on 89320 infected cells. Those infected with Undil. virus are showing good cpe - Those infected with Dil. virus - not as advanced - will harvest the 'Undil.' virus today and possibly the dil virus tomorrow.

Set up ~~Rest XI~~ <sup>Nsi I</sup> digestion on SURE 6 (8.5K?)

2  $\mu$ l RNase A20  $\mu$ l x10 buffer20  $\mu$ l min. prep DNA1  $\mu$ l ~~Rest XI~~ Nsi I

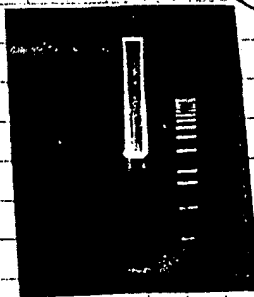
Prepared 2 x 1% Agarose gels - used one for gel purification of Xho I cut ⑧

Used the other for analysis of Nsi I cut SURE ⑥ (8.5K clone)

Prepared 200  $\mu$ l L-Agar and poured 6 Amp<sup>r</sup> plates

Set up transformation of SURE cells using 1  $\mu$ l of religated (Eco RI cut) ② - to try and get the 2<sup>nd</sup> orientation, which should now give an insert band with Xho I digestion - Plated out  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$

SURE ⑥ cut with Nsi I - does not appear to contain the appropriate insert - it was most likely a dimer



Nsi I cut SURE ⑥ - NOT RELEVANT  
ligation of Eco cut SURE ② - it appears there is some religated insert, but the upper band may be reforming CCC DNA. Will check ~30 transformants to try and pick up both orientations

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne W. Kurbili

Recorded by

Dwight K. Clarke

000352

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Meanwhile carried out purification of B9320 Virus (which was showing good cpe) so proceeded with the 6 flasks infected with undil virus.

Clarified at 3K rpm for 10 mins, then put on spin in SW 28 - 1.5 hrs, 4°C 26K rpm - Decanted supernatant and resuspended each pellet in 200 µl PBS. ON on ice.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sgt. W. Kurbale*

Recorded by

*David K. Clarke*

From Page No. \_\_\_\_\_

Checked bacterial transformants - there are numerous colonies - will streak out  
30-60 colonies on Amp<sup>r</sup>, X-gal<sup>+</sup> agar

Went through B9320 purification of cells which had been infected with  
dilute virus (added MgSO<sub>4</sub> and Hepes buffer) - Did a freeze thaw cycle - did a  
cold spin at 3K.r.p.m for 10mins - then pelleted at 26K for 1.5hrs

Set up mini cultures of the 2.25 and 1.5 clones - will inoculate them into  
larger (250ml) broths O/N and do plasmid preps tomorrow.

Collected XhoI cut  
1000bp

Prepared 1% agarose gel and analysed  $\frac{1}{10}^{th}$  of the total XhoI cut (8)  
clone DNA



Injected the previously IN. RSV infected mice, each with 0.3ml of  
a mixture (1:1) C.F.A : RSV B9320 in PBS (virus from one TC150 was resuspended in  
200µl PBS) <sup>I.P.</sup> One mouse died - perhaps from liver damage from the needle + internal

bleeding

125-500 µl<sup>-1</sup>

Total purified DNA = 500 µg - 1 µg

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge W. Kibbe

Recorded by

David K. Clarke

000354



Purification of ② Insert and Vector components  
after EcoRI digestion.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

115

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Set up EcoRI digestions on

Prepared 1% Agarose  
gel for purification  
of all 3 DNA frags.

Prepared 1% Agarose gel for  
purification of bands

① 1 µg pSV DNA

② 100 µl (~2 ng) of ②

Carried out plasmid purifications for 1.5K and 2.25K - 20 min 3K rpm.

Electro-eluted DNA from digestions onto NA45 paper (both digestions looked good)

Run through plasmid purifications for RSV clones 1.5K and 2.25K

Eluted from QIAGEN columns and precipitated.

Resuspended BA320 pellets in PBS (20 µl) following overnight soak at 4°C in ice.

Freeze away at -80°C - May use for 'Tail shot' for mice as a final  
boost for monoclonal Ab production

Took a look at the mice - they don't look v. healthy - but should  
live

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sgt W. Kishik*

Recorded by

*David K Clarke*

000355

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Gel analysis of purified fragments of clone (2).  
Restriction analysis of 1.5K and 2.25 K clones.

From Page No. \_\_\_\_\_

Spin down gel purified Eco cut pcII DNA, and Eco cut  
(2) fragments to assess quality and quantity on a 1%  
agarose gel

Spin down 20µl each of 1.5K and 2.25K and set up EcoRI digest  
of 2.25K clone and Bam/Xho of 1.5K clone

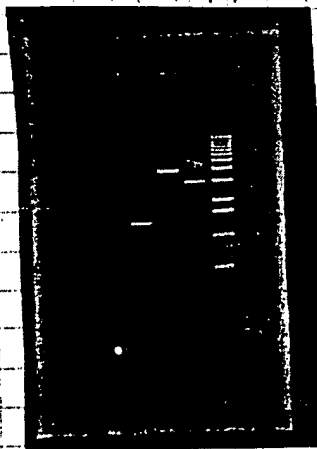
Both XhoI and EcoRI have 5' protruding ends so it should be fairly easy  
to phosphorylate

Insert portion of (2) (EcoRI digest)  
vector portion of (2) (from Eco digest)  
pcII DNA - EcoRI cut  
K8.14.14

Insert portion of (2) = 50ng/2µl

Vector portion of (2) = 100ng/2µl

pcII DNA = 50ng/2µl



Set up S.A.P. RxNs on  
the vector portion of (2) and  
Eco cut pcII DNA, and XhoI  
cut (8) - Then phenol extracted  
and etal potted

Prepared 1% Agarose gel for analysis of 1.5K and 2.25K digests

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge W. Kunkle

Recorded by

David K Clarke

0000356

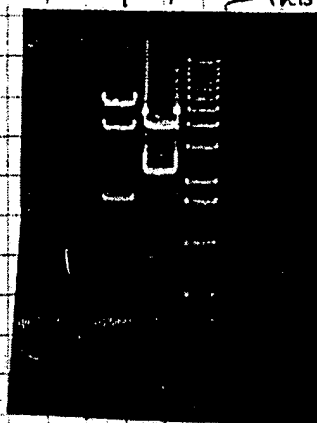
Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

117

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_



Good - both DNAs appear  
to be fine - correct size of  
insert etc.

2.25K digest was done on  
20µl of Eron ppt.

1.5K digest was done on  
20µl of Eron ppt.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Steve W. Kuhl*

Recorded by

*David K. Clarke*

000357

From Page No. \_\_\_\_\_

Ligated 100ng of pCII - Eco cut, gel purified and phosphorylated  
with 50ng of insert from Eco cut (2) (gel purified)

Also ligated 100ng of Vector from Eco cut of (2) (also gel purified  
and phosphorylated) with 50ng of insert.

Set up a Vector only (from Eco cut (2)) ligation as a  
control to test the efficiency of the phosphorylating rxn.

left ligations at R.Temp o/n.

Prepared 9 Amp<sup>r</sup> Agar plates for transformants.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Serge W. Kimble

Date

Invented by

Recorded by

David K. Clarke

Date

TITLE: Transformation of 'SURE' cells with ON ligations  
from page (118)

Project No. \_\_\_\_\_

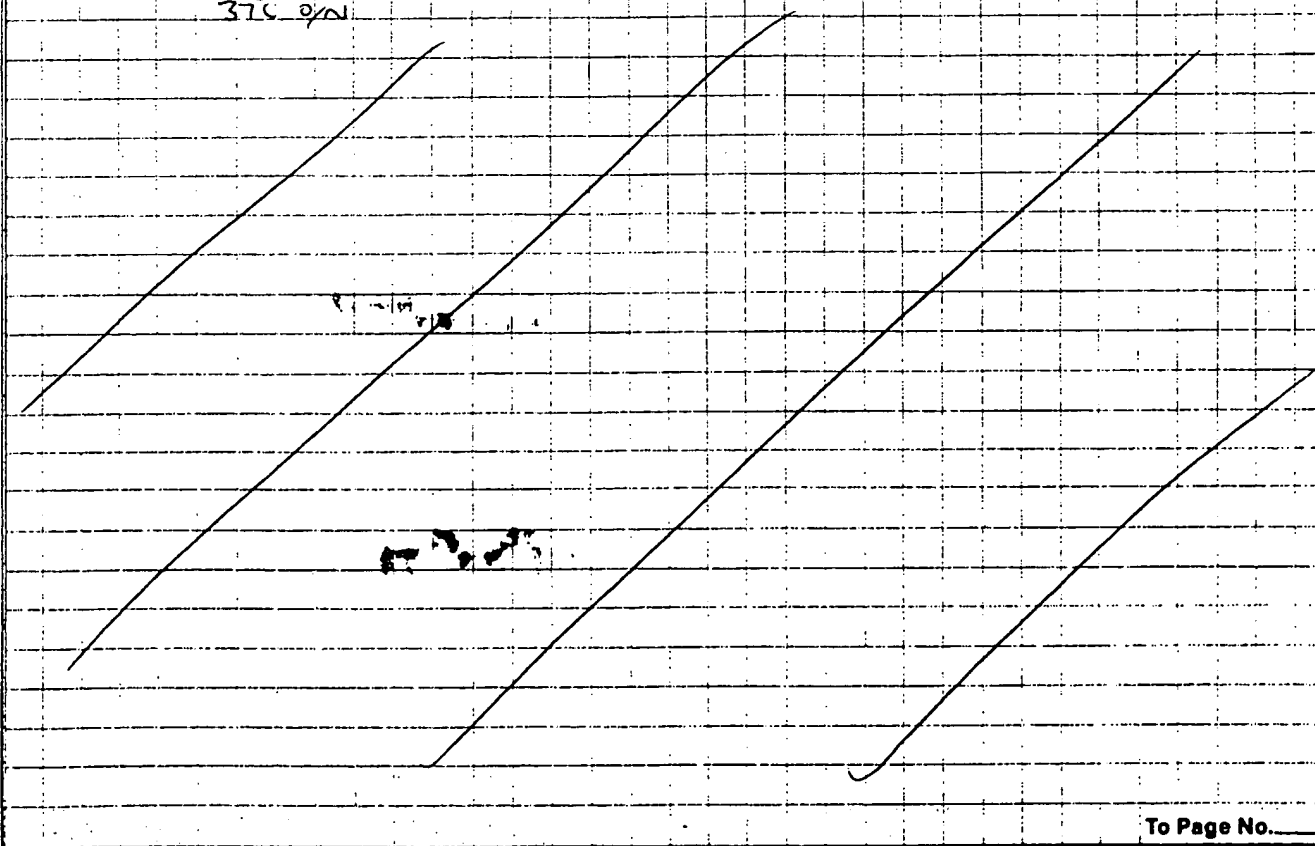
Book No. \_\_\_\_\_

119

From Page No. \_\_\_\_\_

### Set up transformations

Used 'SURE' cells - added 5  $\mu$ l from each ligation mixture  
to 100  $\mu$ l of comp. cells + 1.7  $\mu$ l  $\beta$ -casein - incubated on ice for ~ 1 hr, then  
heat shocked at 42°C for 30 secs - incubated at 37°C after addition of 900  $\mu$ l  
of SOC medium and then plated out 50, 100 and 200  $\mu$ l aliquots from each  
transformation mixture (included X-gal on the agar plates) - Put plates at  
37°C o/n



To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Sege W. Kerbel*

Date

Invented by

Recorded by

*David K Clarke*

Date

000359

From Page No. \_\_\_\_\_

checked transformants - control shows that there is a low background of religated vector - ligations into pCII vector appear to have been most efficient - as it generated the greatest N° of white colonies - hopefully some of these contain Xho/Xcu in the appropriate orientations. Will grow Master plates o/n.

(checked) sequence for insertion of mutations (as markers for recognition of rescued virus)

Selected region of the genome for mutation insertion is the coding and non-coding regions at the end of the 'F' gene.

Page summary!

Set up one culture of other Hsu/Xho clones to look for appropriate orientation.

NON-CODING REGION 5' CCT AAT CAT GTT CTT ACA ATG ~~CTT~~ CTA TCTG CTC ATA GAG

AAC CCA TCT GTC ATT GGA TTT

Pme I changed nucleotides  
GTTTAAAC Pme I

CODING REGION 5' TTG TTA TCA TTA ATT GCT ~~GAT~~ GGA ~~TGG~~ CTC TTA TAC TGT  
L L S L I A V G L L L Y C  
AAG GCC AGA AGC ACA CCA  
K A R S T P

Stu I changed nucleotides  
AGGCCT Stu I

Will order primers as soon as Hsu/Xcu clone is confirmed

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Suzanne W. Kimble

Recorded by \_\_\_\_\_

David K. Clarke

Mini preps of three remaining Hha/Xho clones  
 TITLE: O/N minis of potential re-oriented Xho/Xba clones

Project No. \_\_\_\_\_

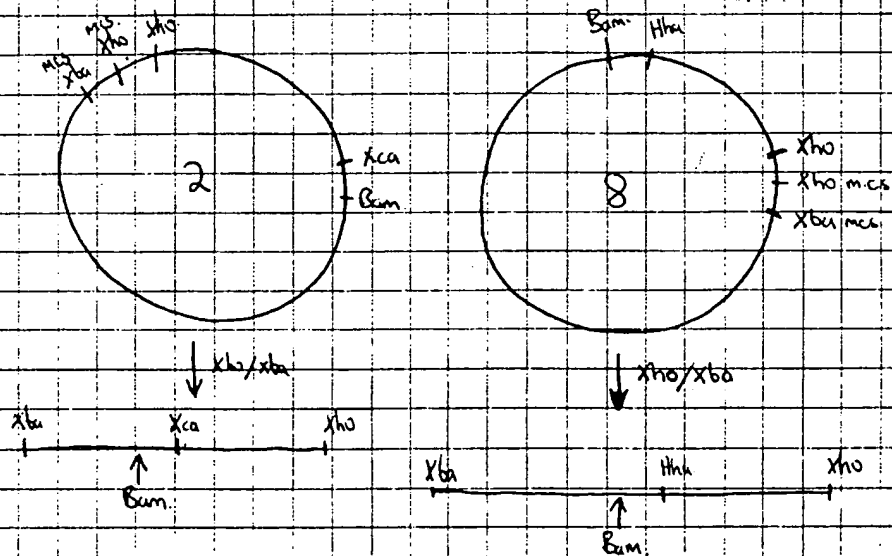
Book No. \_\_\_\_\_

121

From Page No. \_\_\_\_\_

Carried out mini preps on two more Hha/Xho clones to check if they are in the same orientation as ⑧ - set up Xho I digestion as both as a test for orientation

Also set up Xho/Xba I double digests on ② and ⑧ for duplex approach to forming complete 2.3K clone



Can also try flipping ⑧ around (within the MCS - wing the Bcl XI sites on either side of the clone) Will cut, do (fill in) Xca and then purify Bcl XI ended insert prior to religation  
 and Vector portion. Needs to be phosphorylated

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Sege W. Kurbale*

Date

Invented by

Recorded by

*David K. Clushe*

Date

000361

From Page No. 121

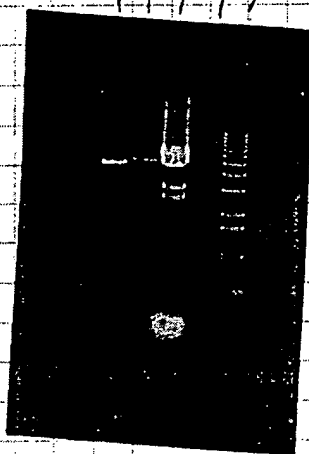
prepared a 1% agarose gel for the analysis of the  $Xho/Xba$  I double digests  
and the  $Xho$  I digests of the  $Hha/Xho$  clones.

⑧  $Hha/Xho$   
⑨  $Xho/Xba$   
⑩ in T/A  
⑪  $Hha/Xho$  in Sure  
⑫  $Xho/Xba$   
⑬ ladder

Looks as though T/A clone  
is unstable after  $ON$  growth

9 SURE - may contain the  
correct orientation (not quite  
enough  $DM$ )

Will try growing: 7, 9, 10, 11, 12, 13  
as  $ON$  cultures on Wed → Thur



Set up  $ON$  minis of  
potential "Flipped"  $Xho/Xba$  clones  
in 'SURE'

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kimbrell*

Recorded by

*David K. Drake*

0000362



TITLE Mini preps to check for "Flipped" Xho/Xca

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

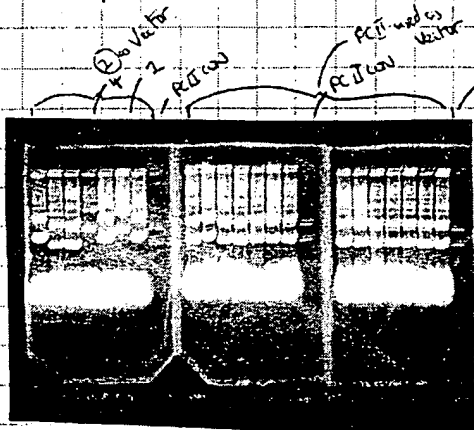
123

From Page No. \_\_\_\_\_

Turned out 21 mini preps to try and identify possible 'flipped' Xho/Xca clones in either the original vector, ② or pCII plasmid.

Did 4 samples from pCII transformation, and 7 samples from ② transformation.

SURE CELLS  
TRANSFORMING



Clones ② and ④ look like possibles - will phenol extract them and then EtBr ppt. prior to Xho I digestion.

Set up 0/2 mini preps of Hhu/Xho clones 7, 9, 10, 11, 12, 13 in an attempt to find the 'other' orientation of DNA in the M.C.S. Stored Xho I digestion of ② and ④ above at -20°C. One - will check on a gel tomorrow.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Suzanne W. Kiehl*

Recorded by

*David K. Clarke*

0000363

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE Gel analysis of possible 'opposite' orientations of Xho/Xba clone

TITL

From Page No. \_\_\_\_\_

From

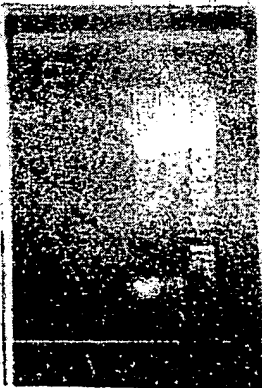
Checked on the Xho clones - they have not grown well - will replate onto a master plate and grow on before redoing mini preps.

Prepared a 1% agarose gel and ran out Xho digested (2)/(4) from yesterday.

Also restreaked remaining Xho/Xba (potential) clones - excluding those already tested.

(2)/(4) - 108 better

Set up O/W mini preps for remaining 'Flipped' Xho/Xba clones (8-14).



Unfortunately neither (2) or (4) are in the correct orientation, although (2) does look as though it contains the correct sized insert.

It may be that the 'G' open reading frame will not permit cloning in the opposite orientation.

Will check the remaining clones to try and find the other orientation.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

With

Steve W. Kemble

Recorded by

David K. Clarke

000364

|| Gel purification of Xho/Xba digested  
TITLE further gel analysis of possible Xho/Xba clones (Flipped)

Project No. \_\_\_\_\_

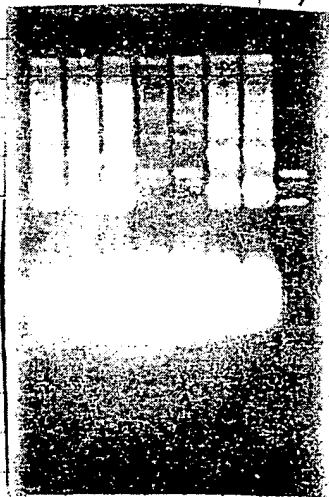
Book No. \_\_\_\_\_

125

From Page No. \_\_\_\_\_

Carried out mini-prep DNA extractions on another (remaining)  
7 'Flipped' clones (3-14) - Run out on a 1% agarose gel  
Also prepared a 'deep' 1% agarose gel for purification of Xho/Xba  
digested ② (Xho/Xba) and ⑧ (Hha/Xho) clones  
11, 10 / a II marker

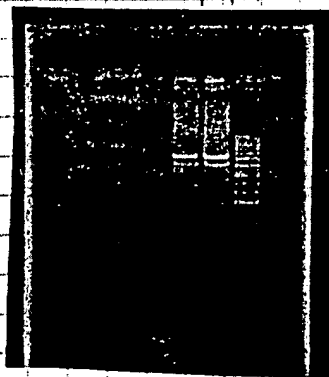
Proceeded with electro elution  
and purification of  
Xba-Xho cut ② and ⑧  
ppt<sup>ed</sup> with a little glycogen



'Flipped' Xho/Xba clones  
10 and 11, look as though they  
contain the correct sized insert  
but what is their orientation?

Proceeded to phenol chloroform  
extract and ETOH ppt

Then set up XhoI digestion  
to see if a 1.3 Kb insert could  
be cut out, indicating the  
correct orientation



Unfortunately neither 10 nor 11  
are in the correct orientation

(Double bands are due to  
blurring during the  
photograph - camera is  
not working properly)

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sege W. Kurbik*

Recorded by

*Daniel K Clarke*

000365

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Analysis of gel purified ②, ⑧ Xba/Xho cut  
Phosphatizing of ⑧

From Page No. \_\_\_\_\_

Completed gel analysis of gel purified Xba/Xho cut ② and ⑧  
 Run 2pl of 20 for each

② ⑧ 1kb ladder

Also set up BamHI digestion  
 of ~~Bac~~ Xba/Bam clone to  
 determine orientation of insert;  
 likewise did a BstUI digest  
 of the 3.6K clone, and will  
 follow up with a Xho I digest  
 tomorrow.



② = 25-50ng Total 200-400ng

⑧ = 50-100ng Total 400-800ng

Proceeded to phosphatase all of  
 ⑧ DNA - Then phenol chloroformed  
 and EtOH ppted

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne W. Kenble

Recorded by

David K. Clarke

000366

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Spin down BstUII cut 3.6K and set up XhoI digestion  
~ 2hrs

Set up 9/11 mini preps for Hha/Xho clones 7, 9, 10, 11, 12, 13  
to see if there were any in the opposite orientation to ⑧

Also set up remaining Xho/Xba mini preps from original cloning

Set up v/v ligation of ② + ⑧ - phosphorylated

Also did ② and ⑧ on their own to check 'background'

Used 4 $\mu$ l of each i.e.

2 $\mu$ l x 10
4 $\mu$ l ② - of 20 $\mu$ l total ~ 80ng
4 $\mu$ l ⑧ - of 20 $\mu$ l total ~ 160ng
9 $\mu$ l H <sub>2</sub> O
1 $\mu$ l Enz
20 $\mu$ l 95°C Temp.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kunkle*

Recorded by

*David K Clarke*

Project No. \_\_\_\_\_

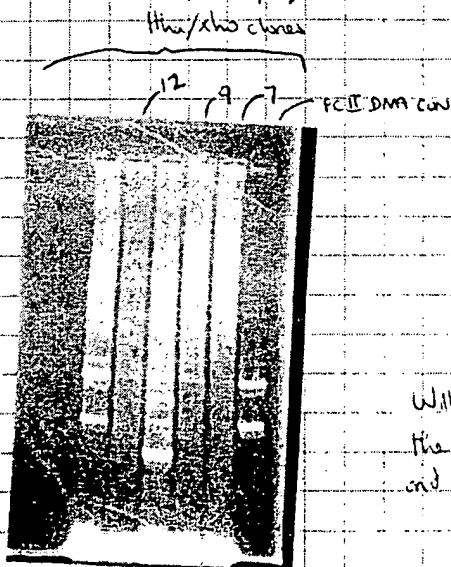
Book No. \_\_\_\_\_

 Transformations with ligated (2/8) Xho/Xba cut.  
 TITLE Mini preps of potential Hha/Xho and Xho/Xba clones

From Page No. \_\_\_\_\_

Checked mini-preps - carried out DNA extractions on those which  
 had grown.

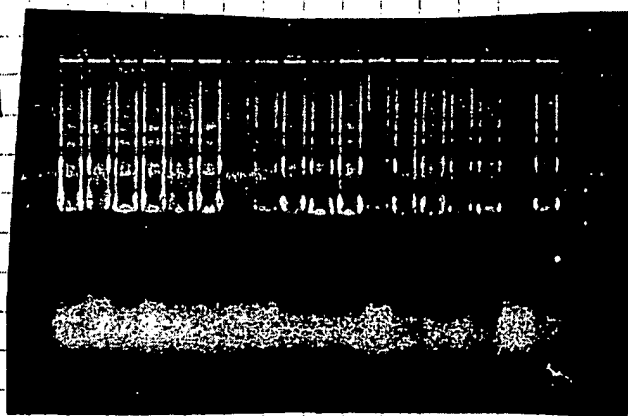
Meanwhile prepared L-Agar for transformations - Did transformations,  
 each with 5  $\mu$ l of DNA ligation mixture (2), (8), (2+8)

Plated out - 200  $\mu$ l/plate

7, 9, 12 look as though  
 they still contain the  
 correct sized insert - on the  
 others the ~~insert~~ plasmids  
 have rearranged compared to  
 their first analysis - following  
 the transformation.

Will phenol extract and EtBr ppt  
 the remaining DNA for digestion  
 and analysis tomorrow

None of these potential  
 Xho/Xba clones appear  
 to contain the  
 appropriate insert



To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Recorded by

0000368

Get analysis of digested 3.6K, 2.7K (Bam/Xba)  
TITLE Master plates for transformants

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

129

From Page No. \_\_\_\_\_

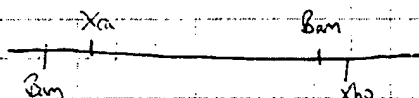
Checked own transformants - strangely many of the colonies are  
blue (in the control (2) - but not phosphorylated (8)) indicating that  
transforming plasmid is deleted in the next region to allow expression of  $\beta$ -galactosidase.  
Will pick some of the white colonies and grow as O/N master plates.

Meanwhile set up XhoI/RnaaseH digests on H/Xho clones 7, 9, 12 from  
yesterday's mini preps to see if any are in the desired orientation.

prepared 1% agarose gel for analysis of digests

⑦ ⑧ Bst/XhoI cut 3.6K  
Xho/Bam cut with Bam  
1KB ladder

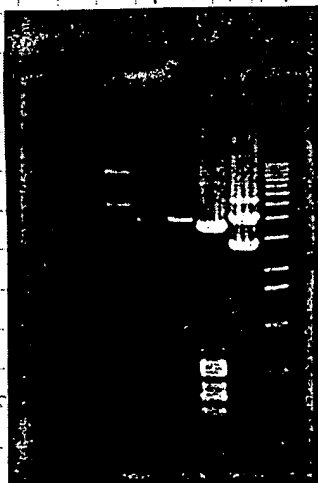
It appears that the Xba/Bam  
clone (2.7K) is oriented as  
follows



It also appears that 3.6K clone

It is not possible to identify  
the orientation of the 3.6K clone

because Bst/XhoI had too many sites in the vector.



Both ⑨ and ⑦ appear to be  
in the opposite orientation to ⑧  
(which is what I need) - Will grow  
these up as a larger scale  
plasmid prep.



Set up own minis of ⑦ and ⑨ for larger scale  
plasmid preps

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sing W. Kuhl*

Recorded by

*David K. Clarke*

000369

Project No. \_\_\_\_\_  
Book No. \_\_\_\_\_

Xba/Bam double digests of xho/xba and 2.7K clones.  
TITLE Digestion of 3.6K clone to determine orientation of insert.

From Page No. \_\_\_\_\_

Checked o/n master plates - the vast majority of clones are expressing  $\beta$ -galactosidase and are blue - however there are a few whites which I will check by Mini-prep.

Checked o/n Minis of ⑦ and ⑨ - they both have grown - stored on ice until ready to set up o/n large cultures for plasmid prep.

Will set up XbaI (Bst 1107I) digestion on 1.3K xho/xba clone,  
XbaI " digestion on 2.7K Xba/Bam clone.

Will do digestions in 50 $\mu$ l and save 1 $\mu$ l for gel analysis.  
Use 2-3 $\mu$ l Bst 1107I for ~2-3 hrs.

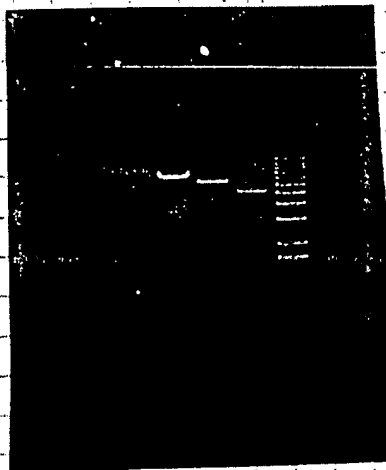
David S. helped (did) the bleeding of the mice - retro orbital - stored the blood

o/n at 4°C to allow clotting before spinning down to collect the serum.

Prepared a fresh stock of 50mg/ml Amp - filter sterilized and aliquotted > 20 $\mu$ l.

Set up o/n plasmid prep cultures for xho/xba

⑦ and ⑨



Good it looks as though all the cuts went as expected.

Completed Bam HI cuts on the 1.3K and 2.7K clones - frozen -20°C til tomorrow.

Phenol chloroform extracted. AvcII cut 3.6K clone - E. coli ppt until tomorrow - will then cut with Bam HI.

To Page No. \_\_\_\_\_

Witnessed & Understood by me.

Date

Invented by

Date

Angie W. Kuhl

Recorded by

David K. Clarke

0000370



Plasmid Preps for Hha/Xho ⑦ ⑧ in orientation to ⑨  
TITLE Bam Digestion of 3.6K clone + gel analysis ON Mini-preps  
of potential Hha/Xho Digest clones

Project No. \_\_\_\_\_

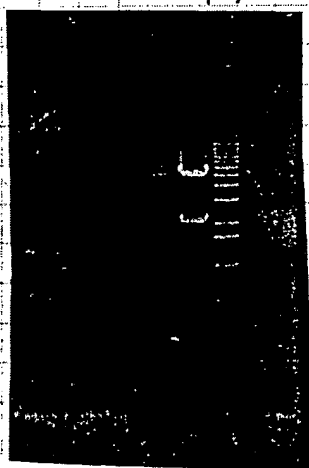
Book No. \_\_\_\_\_

131

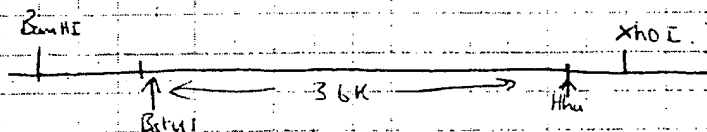
From Page No. \_\_\_\_\_

Set up Bam HI digestion of 3.6K clone (Aur II cut) - Run it out

on a 1% agarose gel  
Aur II/Bam HI cut 3.6K clone  
1Kb ladder



3.6K orientation in vector



Will Hha/Xho I cut ⑨ (next plasmid prep) and

purify the frag (~1Kb) - run then ligate into Hha/Xho I

cut 3.6K (gel purified)

Spin down the clotted Mouse Blood for serum in the 'eppendorf' - then collected as

much serum as possible from each - aliquotted and froze at -20°C - proceeded to let the blood clot further before purifying again for more serum

Run through plasmid preparations for Hha/Xho clones ⑦ and ⑧ - stored as an etOH ppt at -20°C

Set up 20 q/w mini preps of potential Hha/Xho clones - included Kanamycin + Amp in half of them, just Amp in the other half

To Page No. \_\_\_\_\_

Witnessed & Understood by me.

Date

Invented by

Date

*George W. Kuhl*

Recorded by

*David K. Clarke*

000371

From Page No. \_\_\_\_\_

Went through mini preps on potential Hha/Kca clones - stored until sufficient time to do digestion analysis (did phage cloning extraction and stored at E1000 ppt stage)

Will cut with Bam HI/PvuII A to check if correct sized insert is there.

Respin the mouse blood to collect remaining serum - freeze serum with '1st round' stuff at -20°C

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sing W. Koh*

Recorded by

*David K. Clarke*

To Page No. \_\_\_\_\_

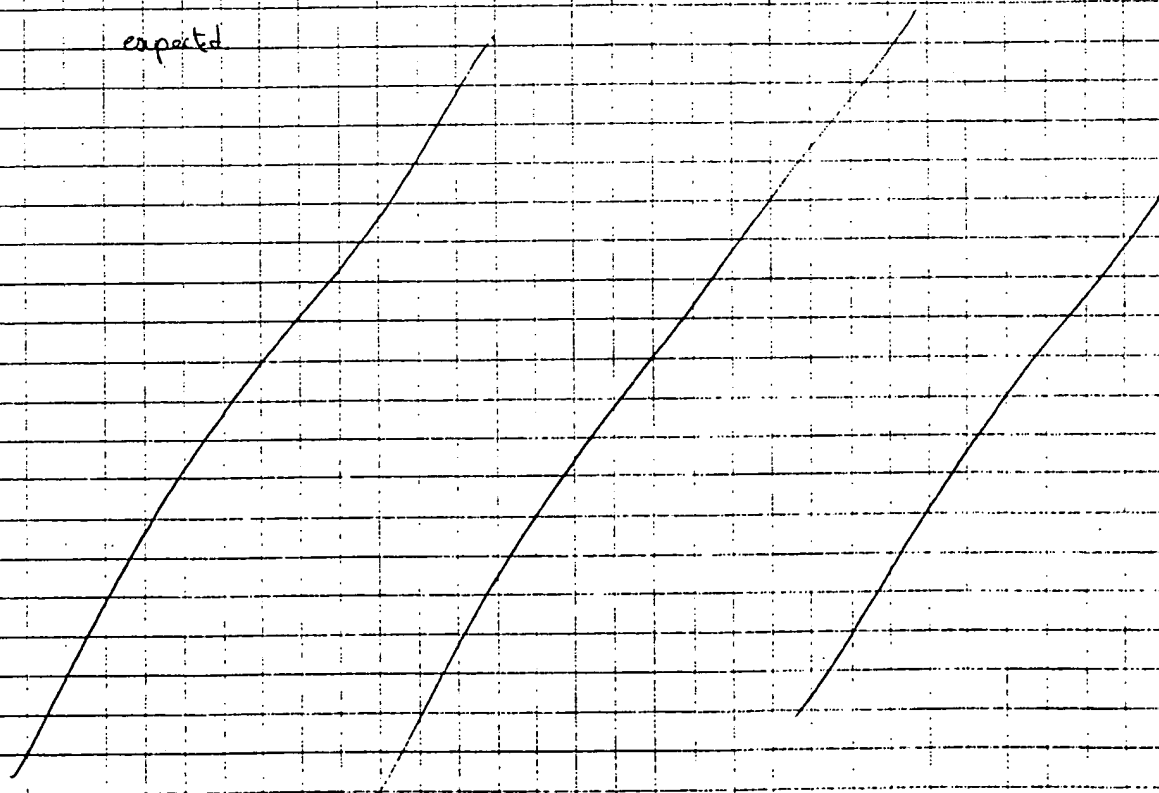
000372

From Page No. \_\_\_\_\_

Set up Bam HI digests on  $\frac{1}{2}$  mini prep material of potential Hha/Xba 'duplex' clones - if the experiment was a success then Bam should give the appropriate 2.3K insert

Also prepared a 1% agarose gel and ran out Bam/Xba cut (2) 1.3K Xba/Xba clone and Bam/Xba cut 2.7K in order to purify the appropriate DNA bands.

The purification went according to plan, and the bands were as expected.



To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Serge W. Kih*

Date

Invented by

Date

Recorded by

*David K. Clarke*

000373

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Analysis of Bam HI cut potential Hha/Xba clones  
 Analysis of gel purified Xba/Bam cut 1.3K and 2.7K clones  
 Analysis of purified plasmids (7) and (9) Hha/Xba

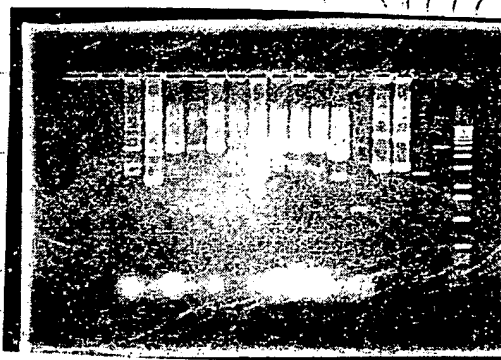
From Page No. \_\_\_\_\_

Prepared a large 20 well agarose gel (1%).

Run out Bam HI digested mini preps of potential duplex Hha/Xba clones.

Also looked at 2 $\mu$ l (1/10) of purified 1.3K clone DNA and 2.7K clone DNA

Also checked 1 $\mu$ l of 40 $\mu$ l of purified plasmid DNA for ~~(1) and (2)~~ Hha/Xba  
 (Plasmid should have (3))



100-200ng of 1.3K Vector

200-300ng of 2.7K insert

Both (1) and (2) look good.  
 DNA conc. in both is 2  $\mu$ g/ $\mu$ l.

None of the Bam HI cut potential duplex clones are showing any promise of containing the Hha/Xba clones.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Invented by

Date

*Sup. W. K. K. K.*

Recorded by

*David K. K. K.*

Phosphataseing of purified 1.3K DNA

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

135

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

Prepared 400ml L-agar to restreak clone banks

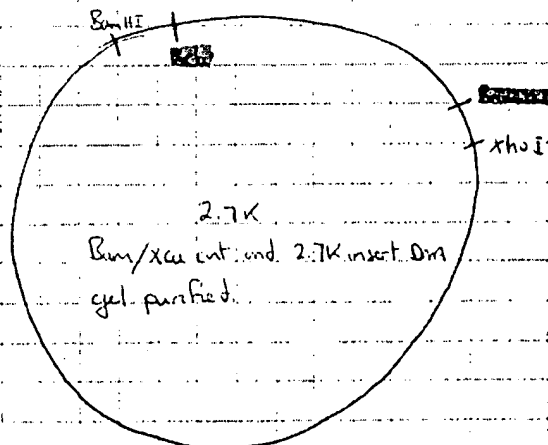
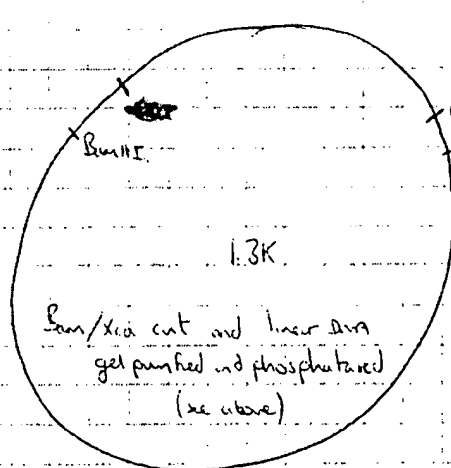
Set up phosphataseing reaction on gel purified 1.3K DNA (vector) - 1hr at

37°C in 30µl - added a fresh 1/2 µl of enzyme after the 1<sup>st</sup> 1/2 hr of incubation

8µl DNA  
3µl x10  
18µl H<sub>2</sub>O  
1µl Enz  
30µl

Then phenol chloroform extracted - added 1µl of glycogen

Streaked out entire clone banks - incubated overnight at 37°C



To Page No. \_\_\_\_\_

Witnessed & Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

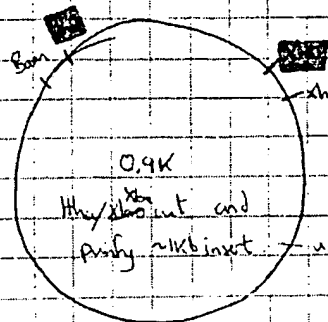
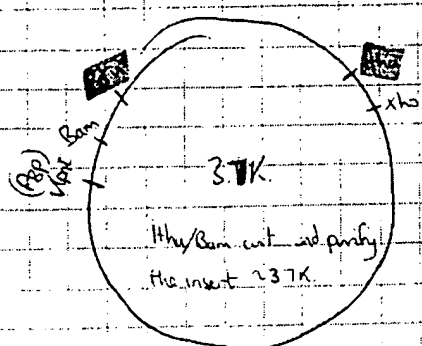
*Shirley W. Kibbe*

Recorded by \_\_\_\_\_

*David K. Clarke*

0000375

From Page No. 135



Unfortunately there are many other I-sites (31) in the vector!!

↓ Phosphatase thoroughly

Then titrate in increasing amounts of unphosphatized 3.7K DNA insert to find optimum ratio for a given amount of 3.7K added - purify the appropriate 4.7K DNA species? - trim with XhoI and ligate into Xho/Bam cut vector

(May want to electroplate into or dialysis bag for larger amounts of DNA)

Should cut ~ 10pg of 0.9K = 2pg of insert  
~ 5pg of 3.7K = 2pg insert

incubate 3.7K insert with a  $\times 5$ ,  $\times 10$  and  $\times 100$  molar excess of phosphatized

0.9K insert - sample at 5min, 30min, 1hr, 2hrs at 37°C

Begin with a  $\times 10$  molar excess of 0.9K clone (phosphatized)  
(need at least as much of 3.7K to give a molar equivalent to 0.9K DNA)

for setting of 0.9 will need 2pg for a 1:1 ratio or 200pg for a 1:10 ratio

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sam W. Kibbe

Recorded by

Marvin K. Clarke

0000376

TITLE

From Page No. \_\_\_\_\_

Prepared a 1% agarose gel for analysis of 1.3K DNA - gel  
 purified and phosphatized - loaded  $\frac{1}{5}$ " (2 $\mu$ l) of total DNA.

Stored clone isunks at 4°C after O/N growth at 37°C

Resurrected JC9387 - the colonies were very old though - so it may not grow - may  
 need to go back to a stab.

1.3K vector - Gel purified and phosphatized  
 1KB ladder

Will also try to join the 0.9K  
 and 1.3K clones by the following  
 mechanism.

① Xho I / ~~Not~~ cut  
 ⑦ or ⑨ 0.9K clones - and  
 gel purify the fragment.

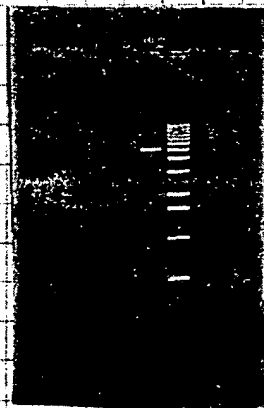
② Xho I cut 1.3K and gel  
 purify - then phosphatase

③ Ligate and transform - look for  
 2.3Kb insert with the Xho digests.

There is between 50 and 100ng  
 of DNA in 2 $\mu$ l of phosphate  
 1.3K vector. Will ligate 50ng  
 of insert 2.7K to 1.3K vector  
 (i.e. 3 $\mu$ l vector)

Ligation RAN

3 $\mu$ l Vector (1.3K) - 50ng  
 3 $\mu$ l Insert 2.7K 50ng  
 2 $\mu$ l x 10  
 11 $\mu$ l H<sub>2</sub>O  
 1 $\mu$ l Enz  
 20 $\mu$ l O/N at 12°C



Prepared analysis tubing for electrophoresis purposes

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Steve W. Kuhl

Recorded by

David K. Clarke

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Transformation with 1.3K/2.7K ligation mixture.  
Digestion of 0.9K and 3.7K clones

From Page No. \_\_\_\_\_

Prepared 300ml L-Agar for plates to do transformation with 1.3K/2.7K ligation mixtures.

Set up  $Hin$  P1 digestions of ~10 $\mu$ g of  $Hha/Xho$  (8) and 3.7K clones in 100 $\mu$ l - used 8 $\mu$ l Enzyme - digested for 4hrs at 37°C

10am  $\rightarrow$  2pm

NB The  $Xho/Xca$  clone would not grow at 37°C on an agar plate - so could not maintain it as a bacterial stock - similarly for one of the 3.6K clones (However have DNA (plasmid) for both clones - so should be able to transform more sure cells)

Redesigned 3.7K/0.9K splicing experiment

- ① will continue with  $Xba$  I cut of 0.9K plasmid (after  $Hin$  P1 cut)
- ② will recut 3.7K with  ~~$Hind$  III~~ and  $Xho$  I Behringer - NEB  
 $Kpn$  I - use  $\epsilon$ -protease Asp 718 or Acc 65 I
- ③ will purify both fragments from ① and ② above by electrophoresis.
- ④ will phosphatase both fragments then trim 0.9K with  $Xho$  I and 3.7K with  $Hin$  P1 (and re-purify if necessary)
- ⑤ Ligate 3.7K and 0.9K DNAs which should ligate predominantly at the  $Hin$  P1 site - Purify if possible - trim with  $Bam$  and  $Xho$  I
- ⑥ Recombine into  $Bam$  and  $Xho$  I cut and phosphatase vect. To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sing W. Khe

Recorded by

David K. Clarke

000378



ON minis of potential Xho/Bam 4K clones  
TITLE XhoI Digestion of ⑦ Hha/Xho

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

139

From Page No. \_\_\_\_\_

Checked bacterial transformants - there are many 'revertants' which are blue - indicating that many of the clones have undergone some type of rearrangement - there are also white clones which will pick and grow as mini preps.

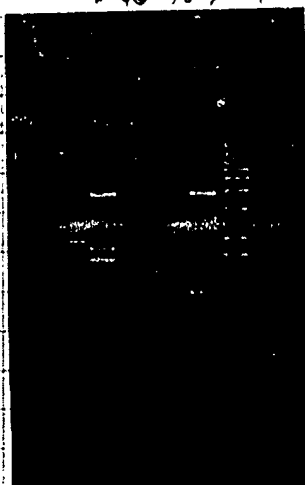
Set up xhoI digestions on ⑦ Hha/Xho and 13K clones in 50 $\mu$ l, 2 $\mu$ l enzyme, ~ 2hrs 37°C

Heat inactivated Mase serum at 56°C for 30 mins - Refreeze at -20°C

Prepared a 1% Agarose gel and run out 2 $\mu$ l (of 50 $\mu$ l) from XhoI digestions above, and from yesterday's digestions 3.7K HindIII/XhoI and HinPI/XbaI cut 0.9K clone

Screened up - there are 3 HindIII sites in the 3.7K clone - although it does confirm the size of the 3.5K clone

Set up 34  $\mu$ l minis of potential Xho/Bam clones



Everything else looks okay

although HinPI/XbaI cut ⑨ has one partial product at ~ 1.2K. However the desired product is there at 0.9K.

XhoI/RspT18 or Acl65I

Will recut 3.5K homodup (and purify XhoI cut ⑦ and 1.3K fragments on SAT - use desalination into dialysis tubing.

To Page No. \_\_\_\_\_

Witnessed & Understood by me.

Date

Invented by

Date

Angus W. Kelle

Recorded by

David K. Clatsh

000379

O/N mini preps had not grown - the incubator had risen to  $42^{\circ}\text{C}$  - too hot  
So set up two master plates of remaining 'white' colonies - will check later  
this evening to see if they have grown sufficiently for  $4^{\circ}\text{C}$  storage, or continued  
growth at R.Temp. o/n.

Set up Apo 718 (Tissachizomer of Kpn I) digestion of 3.7K clone in 40  $\mu\text{l}$   
using 2  $\mu\text{l}$  Enzyme for  $\sim 2\text{hrs}$  at  $37^{\circ}\text{C}$  - will then phenol extract - ppt, and  
set up Xho I digestion - in 40  $\mu\text{l}$  with 2  $\mu\text{l}$  Enzyme  $\sim 2-3\text{hrs}$ .

Meanwhile prepared a 1% Agarose gel and run out Xho I digested 1.3K clone,  
and 0.9K clones - cut out appropriate gel slices - eluted into  
dialysis tubing and then collected and phenol chloroform extracted, and EtOH ppt<sup>d</sup>  
with glycogen. - Will phosphatase the 1.3K DNA, and then analyse both  
purified DNAs to quantitate prior to ligation and transformation.

Checked Master plates (see above); they have grown sufficiently to store at  
 $4^{\circ}\text{C}$ . Will set up mini preps on sun. evening.



From Page No. \_\_\_\_\_

Set up SAP reactions on the following Electro-eluted DNAs① XbaI/Asp718 cut 3.7K DNA② XbaI/HinPI cut ⑧ - 0.9K DNA③ XhoI cut 1.13K DNA

} all have 5' protruding ends, which facilitates phosphatasing.

Set up RXNS. in 50  $\mu$ l final vol.5  $\mu$ l x10 buff4  $\mu$ l H<sub>2</sub>O1  $\mu$ l Enz50  $\mu$ ladded an extra  $\mu$ l of enzyme after 30mins at 37°C and incubated for a further 30mins.Then phenol chloroform extracted and EtOH ppt<sup>d</sup>.Set up one mini preps of potential Xho/Bam clones X 34Will run them alongside the 3.7K 2.7K Xba/Bam clones to identify possibilities

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Steve W. Kibbe

Date

Invented by

Date

Recorded by

David K Clarke

TITLE

Mini prep analysis of potential xho/Bam clones.  
Next test on mouse antisera.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

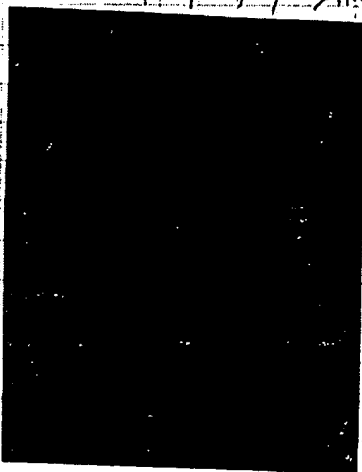
143

From Page No. \_\_\_\_\_

Carried out 34 mini-preps to search for potential xho/Bam clones -

Will run gels later today

Prepared a 1% Agarose gel for analysis of

Gel purified - phosphorylated DNA  
3.7K Ag 718 of  
1.3K xho/Bam cut  
③ xhoI/Bam cut  
⑦ xhoI cut  
- 1Kb ladder

Quantitation

- ① ⑦ = 100ng = 2µg  
 ② ⑧ = 50ng = 1µg  
 ③ 1.3K = 100ng = 2µg  
 ④ 3.7K = 50ng = 1µg

Meanwhile Run RSV neutralisation test

1) Diluted R9320  $V_6$  DCS  $V_4$  32°C  $10^{-1}$  (TCID<sub>50</sub>  $10^{5.5}$ ) to give 50 TCID<sub>50</sub>  
 per 100µl in MEM containing 10% fetal G.Pig. Comp

2) Make serial 2-fold dilutions of each mouse antiserum  $\frac{1}{10}$  to  $\frac{1}{160}$  (after  
 diluting serum initially  $\frac{1}{5}$  - Used 10% G.Pig serum in MEM for dilutions.

3) Added 100µl of diluted virus to each eppendorf containing A serum. Then  
 (Included a no serum con.) incubated at 37°C for 2.5hrs

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge A. Kelle

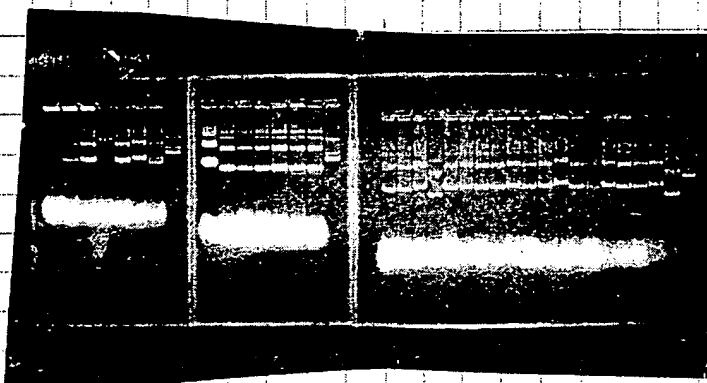
Recorded by

David R. Clarke

000383

From Page No. 143

Meanwhile, carried out a TCID<sub>50</sub> on diluted virus, to verify titer.  
(see official protocol on p. 145)



Unfortunately none of  
the 34 mini-preps show  
the correct sized insert.

Should try transforming JC-cells - call Gallaway for  
mini prep advice.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Invented by

Date

*Samy W. Kheir*

Recorded by

*David K. Clarke*

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

## RSV NEUTRALIZATION ASSAY

1

## MATERIALS

## • VIRUS GROWTH MEDIUM

500 ml Eagle's Minimal Essential Medium  
 5 ml glutamine (200 mM)  
 5 ml Pen-Strep (10k U + 10k µg/ml)  
 5 ml fetal bovine serum

## • VIRUS

Thaw frozen amp of titrated virus at 37°. Immediately dilute in virus growth medium so that there are approximately 100 TCID<sub>50</sub> virus particles per 100 µl. Guinea pig complement may be added (final concentration of 10%). ← MA Bioproducts Cat # 30-456J 5ml #28.35

## • ANTIBODY

The neutralization assay can reliably detect as little as 0.1 µg/ml of antibody. At antibody concentrations greater than 100 µg/ml, non-specific "neutralization" occurs. Therefore, you should dilute test purified antibodies to a concentration between 20 µg/ml and 100 µg/ml. If you are testing unpurified polyclonal sera, make stock dilutions of 1/5 so that the final concentration of the first dilution tested is 1/10.

## • CELL MONOLAYERS

Assay is performed using TC96-well cell monolayers which are about 90% confluent and 2 to 3 days old.

## • TC96 INCUBATION PLATES

## PROTOCOL

1. Add 100 µl of virus growth medium to wells of incubation plate.
2. Add 100 µl test antibody to first well, and make serial two-fold dilutions by transferring 100 µl across the plate. Test antibody dilutions should be set up in duplicate.
3. Add 100 µl of diluted virus to each well containing 100 µl of diluted antibody. Incubate virus-antibody mixture at 37° for 60 minutes.  
 NOTE: Make sure that you save a little of the diluted virus — you need to do a TCID<sub>50</sub> quantitation of your diluted virus in order to confirm that you actually added about 100 particles per well. This is called the "back-titration", and is crucial for interpreting your neutralization assay results.
4. At the end of the antibody-virus incubation period, flick the cell growth medium out of the TC96 cell monolayers, and replace with the antibody-virus mixture.
5. After incubating for an appropriate period of time (~ 4 to 6 days for RSV), examine each each well for cytopathic effect.
6. Flick medium off, and stain with glutaraldehyde and crystal violet. Rinse with water. Endpoint is highest antibody dilution that provides protection from CPE.

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Serge W. Kuhl

Recorded by \_\_\_\_\_

David K. Clarke

000385

From Page No. \_\_\_\_\_

14

Set up PvuI digestion on purified 3.7K DNA, to remove the Vector portion of the DNA - will repurify from an agarose gel

PvuI/clonform extracted and EcoRI gel<sup>ed</sup> - then set up HpaI digestion to firm one end - freeze at  $-20^{\circ}\text{C}$  - will repurify tomorrow from a 1% agarose gel

Also set up EcoRI digestion on miniprep (31) - to check the extent of inserts etc

Checked in with Stratagene on their Yeast Shuttle Vectors - they are sending out the literature

Set up ON ligation of 1.3K XhoI cut Vector and 0.9K XhoI cut insert (Phosphatized vector)

Used 3 $\mu\text{l}$  Vector  $\sim 100\text{ng}$   
1 $\mu\text{l}$  insert  $\sim 100\text{ng}$   
2 $\mu\text{l}$  x10 buffer  
1 $\mu\text{l}$  enzyme  
H<sub>2</sub>O to 20 $\mu\text{l}$  OK at R.Temp

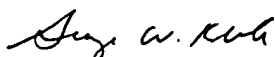
To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date



Recorded by





Transformation with ligated 0.9K/1.3K  
Gel purification of  $HinPI$  hindered 3.7K DNA.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

147

TITLE: \_\_\_\_\_

From Page No. \_\_\_\_\_

Prepared 300ml L-Agar for transformation - Amp<sup>r</sup>.

Set up transformations - 5 $\mu$ l of each ligation mix added to 'SURE' cells

Also set up transformation with 1.3K clone - rcc DNA - (Dried down 1 $\mu$ l from EtOH ppt - resuspended in 5 $\mu$ l H<sub>2</sub>O and used 1 $\mu$ l for transformation.)

Prepared a 1/2 agarose gel for gel purification of  $HinPI$ /<sup>Tand</sup>Asp 718 cut 3.7K DNA.

Called Denise Galloway for hint on mini-props.

It looks as though the  $PvuII$ / $HinPI$  digestion did not go to completion

because there is still some doublet left, indicating that not all the vector was digested

If repeat 3.7K purification will digest with Asp 718/ $Xho$  and  $PvuII$ , then purify and phosphorylate - then ligate with  $HinPI$  and re-purify - Will repeat and see what is obtained with 0.9K/1.3K ligation.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Sing W. Kelle

Recorded by

David K Clarke

000387

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

O/N minis of potential Hha/Xba clones

TITLE Gel analysis of purified HhaI trimmed 3.7K DNA

From Page No. \_\_\_\_\_

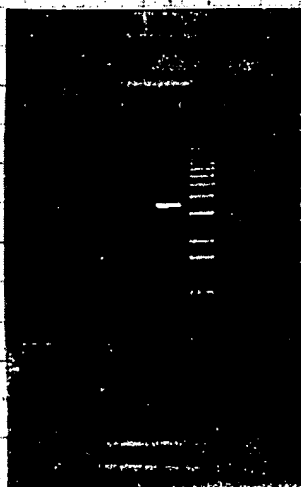
Checked transformants - Virtually all the colonies produced by transforming with the 1.3K ccc plasmid are blue - indicating radical rearrangement of the 1.3K DNA insert. There may be a couple of white colonies which will be colony purified. There are some white colonies on the 0.9K + 1.3K plates - will pick them as O/N mini preps and onto master plates.

Run out 2 $\mu$ l (of 2 $\mu$ l) from re-gel purified 3.7K DNA HhaI trimmed.

HhaI Trimmed 3.7K DNA - Gel purified  
- 1KB LADDER

Colony purified and/or picked the white colonies from the 1.3K ccc transformation.

Picked the white colonies from the 1.3K + 0.9K ligations and set up O/N minis + masters.



There is 100-200 $\mu$ g of DNA of the correct size  $\pm$  2 $\mu$ g of total DNA.

Can now proceed with ligation with 0.9K DNA.

Will use a  $\mu$ g equiv. for each ligation which will give a X.3 molecular ratio of smaller 0.9K DNA.

Need to ~~XbaI~~ XbaI cut (check in gel) and Asp718 cut pDNA II - Gel purify and phosphorylate.

Will start by annealing 100 $\mu$ g of each at 37 $^{\circ}$ C for 1hr  $\rightarrow$  Gel

\* (1 $\mu$ l 3.5K + 2 $\mu$ l 0.9K) \*

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sandy W. Kelle

Recorded by

David K. Clarke

Mini preps of potential Hha/Xba clones.  
TITLE Checking Neutralisation Assays

Project No. \_\_\_\_\_

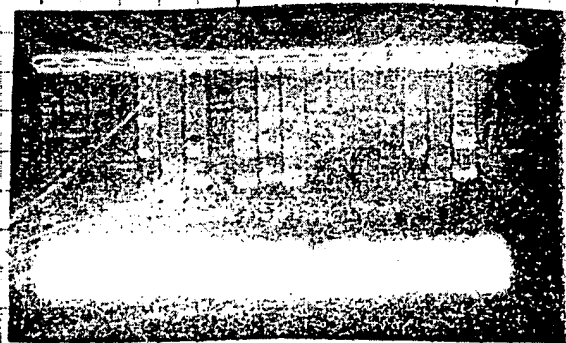
Book No. \_\_\_\_\_

149

From Page No. \_\_\_\_\_

Went through mini-prep procedures for sole potential 1.3K clone, from  
ccc transformation. Also potential Hha/Xba clones from 0.9K + 1.3K ligation  
prepared 1/2 gel, and ran samples with Xho/Xba plasmid as marker.

Checked virus neutralisation assay - it is v difficult to interpret, since the  
complement seems to have adversely affected the cells (and virus?) - The complement  
may not have been suitable for tissue culture work; i.e. it may not be sterile, or  
may contain factors toxic to the virus cells. Will repeat the neutralisation using 3-10  
times the amount of virus, and leave out the complement.



To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Serge W. Kelle

Recorded by

David K Clarke

000389

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE Back to work  
Preparation and planning for the rest of the week

From Page No. \_\_\_\_\_

Back from San Diego todayPhenol chloroform extracted potential Hly/Xca clones and potential 1.3K clone (derived from straight transformation); Error noted1.3K clone = ② superHly/Xca = ⑥ ⑦ ⑧Will be able to digest with BstX1 to determine the size of the insert DNA in these clones - should give two DNA bands; one at 1.7K and one at ~600bpWrote up Bi-monthly report on the RSV project

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

George W. Chub

Recorded by

David K. Clarke

Digestion of mini prep DNAs to check for 1.3K and  
TITLE 2.3K inserts. Ligation of 3.7K and 0.9K DNAs

Project No. \_\_\_\_\_

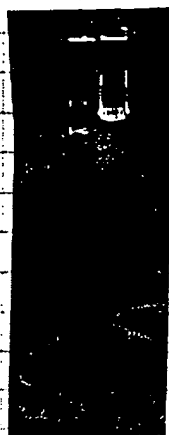
Book No. \_\_\_\_\_

151

From Page No. \_\_\_\_\_

Set up ligation between pushed <sup>(HpaI)</sup> pBp 718/HhaI cut 3.5K DNA and  
purified 0.9K (HhaI/XbaI cut)

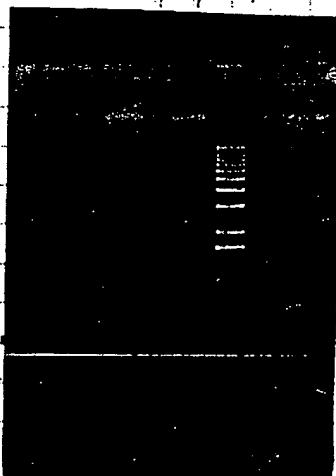
Used ~ 100ng of 3.5K and a X3 molar ratio of 0.9K (~100ng)



(DNA cut so  
all is well)

1ul of 3.5K DNA  
2ul of 0.9K DNA  
2ul x10  
14ul H<sub>2</sub>O  
1ul EN2  
20ul  
1hr, 30min, 1KB ladder

Meanwhile got Lisa to check  
that XbaI is indeed cutting  
⑧ - as should be the case  
to give HpaI/XbaI cut

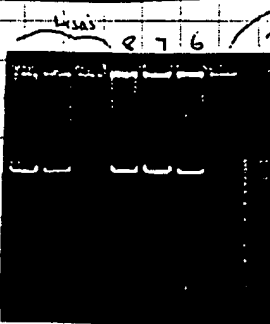


Clearly all of the 3.5K and  
0.9K DNAs are ligating to  
each other, giving high  
molecular bands. There is also a  
little DNA at the 2.3K size,  
which would be what we want.

Also carried out EcoRI digestion  
of potential 1.3K mini prep ②  
DNA and BstXI digestion of  
potential HhaI/XbaI clones

⑥ ⑦ ⑧

double  
Will ~~8~~ the amount of 0.9K DNA  
in the ligation and cut the time  
to 15min at 37°C



1.3K clone is good  
1KB ladder

None of the potential HhaI/XbaI clones  
were good, however the 1.3K  
clone is good, so will make a  
glycerol stock from an overnight  
culture tomorrow.

Witnessed & Understood by me,

Date

Invented by

Date

Sergey W. Khleli

Recorded by

David K. Clarke

To Page No. \_\_\_\_\_

000391

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

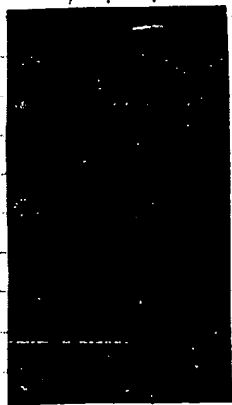
TITLE \_\_\_\_\_

Ligation for 0.9K to 3.5K DNA - modified protocol  
ON Mini prep of (2) - 1.3K for glycerol stock

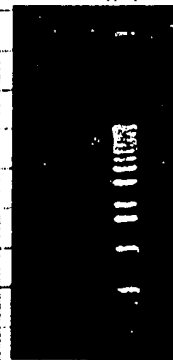
From Page No. \_\_\_\_\_

Tried setting up another ligation between 3.5K and 0.9K DNAs.

This time doubled the amount of 0.9K DNA and halved the incubation time.

1  $\mu$ l 3.5K DNA4  $\mu$ l 0.9K DNA2  $\mu$ l x1012  $\mu$ l H<sub>2</sub>O1  $\mu$ l Enz20  $\mu$ l15 mins at 37°C - sampled  $\frac{1}{2}$  at 7 mins15 mins  
7 mins  
1K8 ladderStill not enough 0.9K DNA - will redo ligation with 8  $\mu$ l of 0.9K DNA1  $\mu$ l 3.5K DNA8  $\mu$ l 0.9K DNA2  $\mu$ l x108  $\mu$ l H<sub>2</sub>O1  $\mu$ l Enz20  $\mu$ l

15 mins at 37°C - sampled at 7 mins

15 mins  
7 mins  
1K8 ladder

Obtained Zymolase from Ying (ICN supplier)

Will proceed by XbaI / Psp718 cutting pII DNA  
(check after 1st cut) and gel purification.Set up ON 1.3K (2 super) from remaining Mini prep  
culture (from last week)

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge W. Kibbe

Recorded by

David K. Clarke

To Page No. \_\_\_\_\_

0000392

Long term storage of 1.3K clone; Double digestion  
of pc II Vector (XbaI/Asp 718)

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

153

From Page No. \_\_\_\_\_

Checked on growth of 1.3K clone 'Super' (2) - it had grown so set  
up glycerol stocks (0.15ml glycerol + 0.85ml o/n culture) and froze away  
at  $-90^{\circ}\text{C}$ .

Set up XbaI digestion of pc II DNA (~2µg) in 20µl final vol with 1.5µl of  
enzyme. - Prepared 1% gel for analysis of 100ng ( $\frac{1}{20}^{\text{th}}$ )

Stricked out 1.3K clone from o/n mini prep onto agarose gel agar Amp<sup>r</sup>  
and grew o/n at  $37^{\circ}\text{C}$

\* Lisa carried out a \*  
mini prep extraction on 1.3K  
o/n culture + EcoRI  
digestion - to ensure  
that the o/n culture does  
indeed contain the  
correct insert.

! gel worked - it will have  
to be repeated.



There are trace amounts of uncut acc  
and open circle plasmid; probably due to  
Dam/Dcm methylation of the XbaI site.  
However will go ahead with  
gel purified XbaI/Asp 718 cut.

plasmid cloneform extracted XbaI cut pcII DNA - etOH pptd and then set up

Asp 718 digestion for ~ 3hrs in 20µl with 1.5µl Enzyme - froze at  
 $-20^{\circ}\text{C}$  prior to gel purification.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Shay W. Kibbi

Recorded by

David K Clarke

- 000393

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE \_\_\_\_\_

Gel purification of Asp718/XbaI cut pCII DNA.  
Patent Writing.

From Page No. \_\_\_\_\_

Took one 1.3K clone from 37°C - it had not grown well - restreaked  
some of the colonies on a fresh Amp<sup>r</sup> plate and grew again at 37°C.

Prepared a 1% Agarose gel for gel purification of XbaI/Asp718 cut pCII DNA.

Run out DNA - excised DNA band and electro eluted - Phenol chloroform extracted  
and etOH pptd with some glycogen.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,,

Date

Invented by

Date

*George W. Kibler*

Recorded by

*David K. Clarke*



TITLE: Ligation of (0.9K+3.5K) into gel pure pET Dna.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

155

From Page No. \_\_\_\_\_

Patent writing!

Rechecked the T.C. Room!!  
tomorrow evening

Checked temps + H<sub>2</sub>O levels - will check CO<sub>2</sub> levels

Task 1.3K @ Super from 37°C incubator - it appeared to have grown fairly well - stored at 4°C.

Set up the 0.9K/3.5K ligation  
Gave Dna at 37°C

→ then added the gel purified pET vector (200ng total added) in XL ligation buffer

7µl 0.9K

1µl 3.5K

2µl XL

9µl H<sub>2</sub>O

1µl Enz.

20µl

ON R Temp

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Shay W. Kibbi*

Date

Invented by

Recorded by

*Daniel K. Clarke*

Date

0000395

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

Transformation with 0.9K + 3.5K + pCII DNA.  
TITLE Neutralization Assay with mouse serum.

From Page No. \_\_\_\_\_

prepared 1-agar - amp<sup>r</sup> - 300ml to streak out transformants

Have prepared SCE solution (for Yeast cloning protocols)

Sorbitol  
Sodium Citrate  
EDTAAlso prepared 5M K<sup>+</sup> Acetate.

SCE.

Carried out bacterial transformation with 0.9K + 3.5K ligated into pCII DNA

Used 5 $\mu$ l of 0.1M ligation mixture for transformation - plated out  
on Amp<sup>r</sup> Agar containing Xgal and IPTG. - incubated overnight at 37°CAlso plated out 10<sup>-1</sup> diluted transformed cells.Set up Virus Neutralisation assay - diluted R4320 ( $V_{60} \times 5, V_{100} \times 10^{-1}$ )10<sup>-1</sup>  $\rightarrow$  10<sup>-1</sup> (5ml)  $\approx 5 \times 10^3$  pfu/ml. Incubated w/ 100 $\mu$ l ofeach of the 5 mouse antisera - diluted serially two-fold (in w/ 100 $\mu$ l final vol) $\frac{1}{10} - \frac{1}{20} - \frac{1}{40} - \frac{1}{80} - \frac{1}{160}$ 

~ 1hr at 37°C

Also did back titration - to get a TCID<sub>50</sub>

reading for the diluted virus used. Run a No. antiserum control.

Have set up 0.1N 1.3K cultures; one from an agar plate  
one from a broth.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne W. Kelli

Recorded by

David K. Burke.

0/w minis + master plates for potential 35K + 0.9K  
TITLE Clones in pet

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

157

From Page No. \_\_\_\_\_

checked bacterial transformants - there are many blue colonies - but there does appear to be a few whites - will pick them later and set up master plates

Lisa proceeded with mini preps - of 1.3K grown in broth/Agar (The broth derived culture had not grown v. well)

Called Stratagene for yeast stuff - should be here by tomorrow

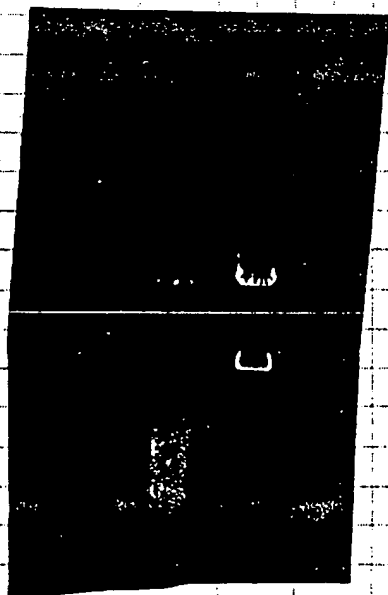
Prepared 40% PEG (4000) solution, 10mM Tris (pH 7.5) - autoclaved

checked in with Gengen on their column for use in plasmid purification from yeast. They suggested dissolving the pellet (after Isopropanol ppt) in T.E then adding Q.B.T. → column

(Use small vol. of T.E and then x10 vol. of Q.B.T)

Lisa's gel shows that the clone does indeed contain the 1.3K insert (After EcoRI digestion)

She will check the clone down grown as a broth tomorrow



To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sing W. K. Khoh*

Recorded by

*David K. Clarke*

0000397

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE O/N yeast culture / Prep<sup>n</sup> for Yeast Cloning.  
Mini preps of potential 0.9K+3.5K in pET vector

From Page No. \_\_\_\_\_

Checked Master plates at 37°C - many have turned blue - will not do these as mini-preps - stored O/N mini preps at 4°C until ready to do mini preps.

Received yeast cloning vectors + host strains from Stratagene - stored strains at -80°C, and Vector at -20°C

Prepared 1 Liter of 0.1M Lithium Acetate - autoclaved

Ordered 2 x 96 well plates for min for neutralization assay (for min)

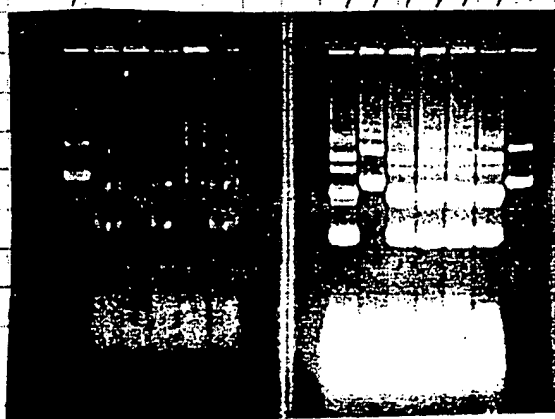
Cured out mini preps of all 'white' clones from master plate - Run out on a 1% agarose gel with 3.6K clone as a marker (stored master plate at 4°C)

prepared YPD broth for growth of yeast cells

Set up O/N

YAH 499 culture in

YPD broth (~3ml)  
at 30°C



No. 11 looks like a good candidate.

A couple of hisai minis may also be okay

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Serge A. Kuhl*

recorded by

*David K. Clarke*

From Page No. \_\_\_\_\_

Checked growth of yeast on culture - it hadn't grown yet - so left at 30°C for a further 24hrs. Meanwhile prepared some YPD Agar for plates in order to have a yeast stock on a plate.

Set up BstUI digestion of mini preps ⑪ + 2 of tisas

Checked the Neutralisation assay - it looks good - most mice on fixers are showing good neutralisation activity; some out to  $\frac{1}{60}$  and  $\frac{1}{160}$  dilution - will make a final reading tomorrow.

Meanwhile plated out YFH44/SW on YPD agar, to get a stock on agar plates.

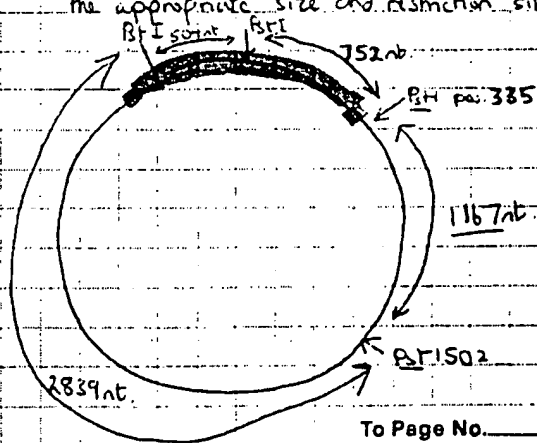
Also continued digestion of ⑪ with Asp718 - ~ 2hrs in 40µl - 1µl Enz

ladder / EcoRI cut 1.3K / Bst cut 1.3K

Lisa's digestion of potential 1.3K clone



Gel indicates that the 1.3K clone (super ②) is indeed the correct clone - and has the appropriate size and restriction sites



To Page No. \_\_\_\_\_

Witnessed & Understood by me.

Date

Invented by

Date

*Suzanne W. Kibbe*

Recorded by

*David K Clarke*

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

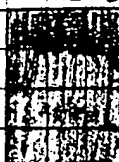
Analysis of double digests Ap718/Bstur on potential  
0.9% + 3.5% clones. Read of Naut Assay

From Page No. \_\_\_\_\_

Checked growth of yeast cells in broth + plate cultures. The broth looks as though it is growing, and there are very, very small colonies on the plate - things should have improved by tomorrow.

Made a final reading on the Naut assay + Back Titration TCID<sub>50</sub>

-1 -2 -3

B9320: V<sub>6</sub> DC5: V<sub>4</sub> 37°C 10<sup>-1</sup>10<sup>3.5</sup>

oops!

The diluted stock which was used in the neutralisation assay contained  $5 \times 10^3$  pfu ml<sup>-1</sup>; 100  $\mu$ l contained  $5 \times 10^2$  pfu

	1/10	1/20	1/40	1/80	1/160
Mouse Number 1	0	0	1	4	16
2	0	0	0	5	15
3	0	0	0	1	3
4	0	0	2	6	15
5	0	1	2	4	7
CON					

OE No plaques visible (1-16 = No. of plaques)

Control cell monolayers were wiped out

Will use mouse No 3 for monoclonal production

prepared a 1% Agarose gel for analysis of

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne W. Kuhl

Recorded by

David K. Clarke

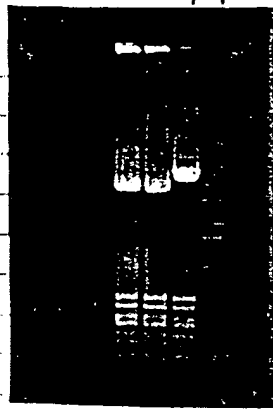
000400

TITLE \_\_\_\_\_

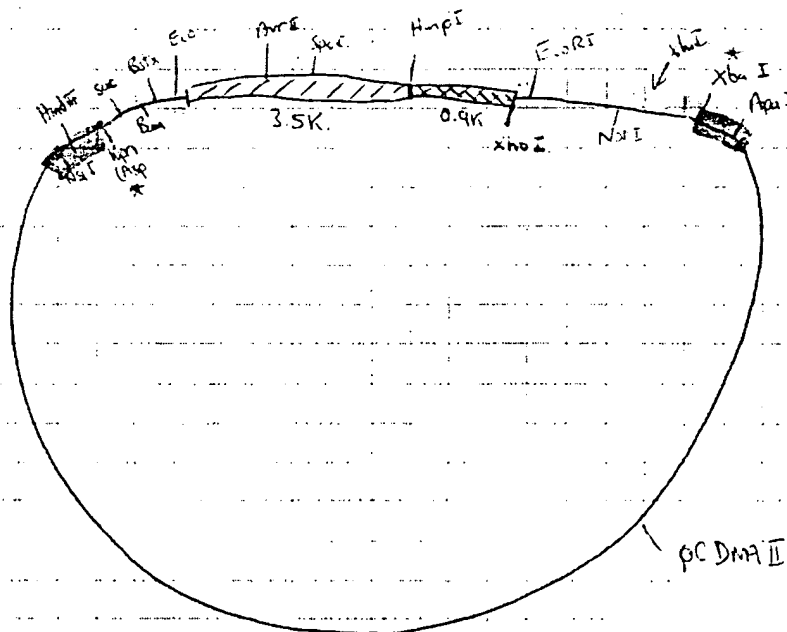
From Page No. 160

Gel shows that clone  
No ⑪ is probably the

0.9K + 3.5K clone - will  
check with further digests.



Structure of MCSite of 0.9K + 3.5K in pcII vector



■ = MCS  
belonging to pcII Vector  
□ = MCS belonging  
to pCR II.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Invented by

Date

Serge W. Kibeli

Recorded by

David K Clarke

000401

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE Patent writing / Yeast cell check

From Page No. \_\_\_\_\_

Checked growth of yeast on YPD agar - colonies are quite large, but  
will leave for another 24hrs, before storing at 4°C

Tidied up Fin. T.C. Room etc.

Rewrote some of Patent application.

Set up new agar plate of ⑪ (0.9K + 3.5K m.p. II)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Seay, W. K. K.

Recorded by

David K. Clarke

000402



TITLE: O/N YEAST CULTURE YPH 499

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

163

From Page No. \_\_\_\_\_

Took bacteria (0.9K + 3.5K in p.c.) from incubator - it had grown quite poorly as expected - and stored at  $4^{\circ}\text{C}$ . Will set up a mini prep tomorrow for long term storage of this clone in glycerol at  $-80^{\circ}\text{C}$ .

Took yeast from  $30^{\circ}\text{C}$  incubator and set up one YPH 499 in  $\sim 2.5\text{ml}$  of YPD broth - Used a very large loopful of yeast cells to inoculate the new culture.

Continued writing Patent material.

Temp check on the T.C. Room incubators.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Shirley W. Kibler*

Recorded by

*David K. Clarke*

000403

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

Neutralisation assay for MAB I-13.  
TITLE Preparation of Competent Yeast Cells YPH 499

From Page No. \_\_\_\_\_

8-45 Inoculated ~100ml of YPD broth with ~1.2ml of OAD culture

Will check O.D. at ~1pm or 2pm vs YPD blank

2pm checked O.D. <sub>600</sub> of culture - it was ~ 0.531 - will check again ~

3-30pm

Meanwhile carried out 'Back titration' on diluted ( $10^{-2}$ )  $V_6$  DCS  $V_4$   $32 \times 10^{-1}$ 

and a virus neutralisation assay using I-13 MAB and a PIV3 HN MAB

MAB DIT:  $\frac{1}{10}$   $\frac{1}{20}$   $\frac{1}{40}$   $\frac{1}{80}$   $\frac{1}{160}$  100 $\mu$ l (~500 pfu) into each dil<sup>n</sup> of AR

Control - No AR

Meanwhile Kathy shot up mouse No. 3 with ~30 $\mu$ l of conc. #320 - final  
boost before harvesting spleen (i.p. injection)

Went through Protocol for preparation of competent yeast cells - harvested

at 0.8-1.0 A<sub>600</sub> - 2.5K/gmins in 50ml glass tubes

Resuspended each pellet in 10ml 0.1M LiAc

Respin

Resuspended each pellet in 5ml 0.1M LiAc and  
incubated at 30°C for 1hr at 800rpm in small  
petri dishesDecontaminated cells, resuspended in 0.5ml 0.1M LiAc  
and stored OAD at 4°C.NB Yeast Cell growth  
took ~ 8hrs to reach  
1.0 A<sub>600</sub>Also: Use YPD broth that has  
been autoclaved for 15mins only

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Dennis W. Kibler

Recorded by

David K. Clarke

000404

Plan for assembly of RSV 5' end (L-end)

TITLE: Transformation of Yeast - LiAcetate - No Glycerol

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

165

From Page No. \_\_\_\_\_

Had forgotten to put 900  $\mu$ l (0.9K + 3.5K  $\mu$ l) in incubator -  
Set it going and will use it to set up 900  $\mu$ l plasmid prep (large scale) for this plasmid  
Went through Yeast Transformation protocol - used 500  $\mu$ l of cells/transformation -  
added 1  $\mu$ g, 100  $\mu$ g, 100  $\mu$ g of pRS416 - plated out cells on YEA Minimal Medium  
(after finally resuspending in 100  $\mu$ l of H<sub>2</sub>O) - Incubated for 48 hrs at 30°C

Plan for assembly of RSV 5' end

- ① a) Digest 3.0K clone with XhoI and PmlI to determine orientation.  
One orientation is established, digest with PmlI and phosphatase, then  
digest with suitable MCS enzyme XhoI or KpnI.
- ② Digest 1.5K clone with SphI and PmlI + (HhaI to remove interfering  
vector bands).  
Purify the DNA on a gel.
- ③ Digest Bam/Sph clone with BamHI to check orientation. One orientation is  
established. cut with SphI - phosphatase, then cut with BamHI and  
HhaI (to remove vector DNA) and purify the appropriate band.
- ④ Find conditions for 3-way ligation - use 2:1:2 ratio (molar) initially, and include some BamHI and either XhoI or KpnI in the ligation to fine tune the 3-way ligation - analyse aliquots on a gel at 5, 12, 20, 30 min at 37°C.
- ⑤ ~~the~~ Ligate 'best' material into Bam and XhoI or KpnI cut and  
phosphorylated and gel purified Vector.

Witnessed & Understood by me,

Date

Invented by

Date

Sevgi W. Kulu

Recorded by

David R. Clarke

To Page No. 166

000405

From Page No. 65

Used mini prep broth ⑩ (0.9K + 3.5K in pCII Vector) to inoculate a 250ml broth  
for preparation of large stock of this plasmid.

Freeze down 2 vials of ⑩ mini prep in glycerol (15%) at  $-80^{\circ}\text{C}$

Witnessed &amp; Understood by me,

*Serge W. Kuhl*

Date

Invented by

Recorded by

*David K Clarke*

Date

To Page No. \_\_\_\_\_

000406

Orientation of 3K and 2.25K clones by Restriction Digest. Project No. \_\_\_\_\_

TITLE: \_\_\_\_\_ Book No. \_\_\_\_\_

167

From Page No. \_\_\_\_\_

Set up *Xho*I digestion of 3K clone and *Bam* HI digestion of 2.25K clone in order to determine orientation of the clones, relative to the MCS.

Setup digests in 40  $\mu$ l, with 1.5  $\mu$ l enzyme for + 2-3 hrs at 37°C.

Phenol Chloroform extracted *Xho*I digestion of 3K clone and EtOH ppt<sup>ed</sup>

Spun down and set up *Pml*I digestion - 30  $\mu$ l, 1.5  $\mu$ l Enzyme ~ 1.5 hrs 37°C

Prepared a 1% Agarose gel, but left until tomorrow to run out samples.

Lanes run through plasmid purification for (0.9K + 3.5K in p. II) - Stored in EtOH - will dry down 5  $\mu$ l tomorrow and run on the gel

Checked Yeast Transformants - there appears to be some colonies starting on the 1  $\mu$ g transformation plate. Will check again tomorrow. May need to investigate electroporation as a means of transformation - to boost efficiencies.

Went over monoclonal preparation protocol with Dave S.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Se. W. K. K. K.*

Recorded by

*Harold K. Clarke*

000407

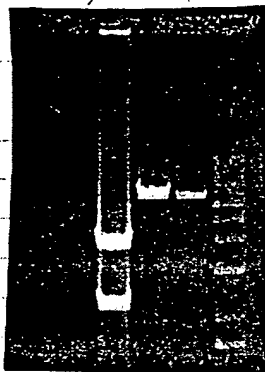
From Page No. \_\_\_\_\_

checked Yeast transformations - there are 20-30 colonies on the 10g plate; not yet visible on the 10mg and 10ng plates.

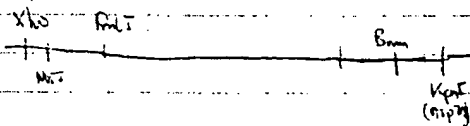
prepared 1/2 agarose gel and ran out digests from yesterday

1KB buffer / Xho cut 3K / Xho/Pml I cut 3K / Bam cut 2.25K / Locus plasmid prep

Locus plasmid prep did not give anything



3K orientation is :-



2.25K Orientation is :-



(3hr procedure) Went through Monoclonal antibody preparation protocol - Spleen was on the left side of the mouse and was very enlarged. Kim demonstrated teasing out the cells with a bent syringe needle. Used RPMI medium, instead of DMEM medium.

\*Preparative\*

Will digest 3K with Pml I - then phosphatase and digest with Kpn I and purify 3K DNA.

2.25K (3hr) Will digest 2.25K with Sph I - then phosphatase and digest with Xho I and purify 2.25K band.

Will digest 1.5K Sph I + Pml I + (Hha I) to remove vector and purify 1.5K band.

Checked I-B MAB neutralisation assay - No neutralisation was apparent in any of the wells - Virus may have drifted; Virus strain may not be B9320 - MAB may not be active, or may be incorrect.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sgt. W. K. K.

Recorded by

David K. Clarke

0000408

Bulk digestions/Phosphatasing of 3K, 1.5K and 2.25K  
 TITLE: Restricting clone banks

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

169

From Page No. \_\_\_\_\_

prepared 400ml L-Agar (amp<sup>r</sup>) - poured ~ 12 plates in order to restreak  
 clone banks

Checked Yeast Transformation again; there are ~ 52 colonies on the  
 1<sup>st</sup> transformation, but none at 400ng or 100ng. Will streak master plate with  
 some of the transformants, in order to do some mini preps.

Set up large scale digestions on 1.5K, 3K, and 2.25K ~ 3hrs at 37°C.

3K - pml I in 50µl Spl. Enz  
 2.25K } Spl I in 50µl Spl. Enz each.  
 1.5K }  
 Lisa is preparing solutions  
 for Yeast Electroporation.

Will phenol/chloroform extract and ppt prior to phosphatasing  
 treatment. Will digest 1.5K with Pml I without phosphatasing (then Hha I).

⊗ Talked to Kathy about Monoclonals:

MAB #	Antib titer	B oant titer
1112	200	5,120
1243	120	5,120

} these two would be  
 possible for selection  
 against 'B' Helper.

Would also be beneficial to get a Mab(s) against 'G' of A2 which would  
 bind but not neutralize.

Set up Pml I digestion of 1.5K in 50µl - Spl. Enz ~ 3hrs 37°C  
 Also set up phosphatasing reactions on pml I cut 3K and Spl I cut 2.25K  
 in 100µl with 4µl + 3µl of phosphatase (SAP)  
 Phenol chloroform extracted all 3K and ppt<sup>d</sup> in EtOH with <sup>Glycerol</sup>  
 To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Sig. W. K. Chae

Recorded by

David K. Chae

000409

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

H<sub>inf</sub>I digestions of 2.25K and 1.5K clones to  
remove vector portion of plasmid.

From Page No. \_\_\_\_\_

Checked Yeast and Bacterial master plates - will leave until later  
to store at 4°C. The yeast had grown fairly well - so over growth on  
an agar plate should be doable - O/RX minis should probably be set  
up around mid-day for the following morning.

Set up H<sub>inf</sub>I digests on 2.25K and 1.5K DNAs in 50µl with 5µl of  
enzyme - incubated at 37°C for ~3 hrs.

Phenol Chloroform extracted 2.25K DNA and EtOH pptd.  
Stored 1.5K DNA frozen (in 50µl)

Left Yeast at 30°C until tomorrow

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sey W. Kuhl

Recorded by

David K. Clarke.

To Page No. \_\_\_\_\_



From Page No. \_\_\_\_\_

Set up  $XhoI$  digestion of 2.25K DNA } in 50 $\mu$ l - Spl Enzyme  
 $KpnI$  digestion of 3K DNA }

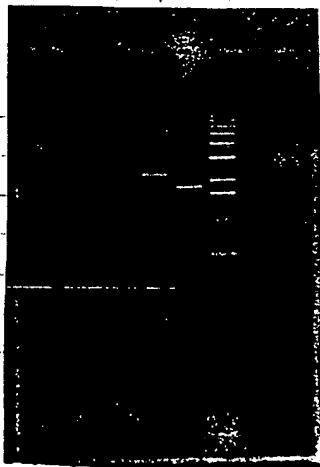
Prepared a 1% gel for analysis of digests (after ~2hrs)

Set up 0M yeast cultures in YPD broth for preparation of Competent cells (Error - tried mini preps of transformants in YPD - Need Drop-out Ura<sup>-</sup> broth)

Prepared 12ml drop-out broth - unthawed for 15mins

Set up 0M culture of (0.9K + 3.5K in pCD)

Only the 2.25K fragment has behaved as expected.



1.5K is not migrating correctly (it is too big) Need to cut 1.5K plasmid with

- 1)  $PstI$
- 2)  $SphI$

3K is not migrating correctly (it is too big). Need to cut 3K plasmid with

- 1)  $PstI$
- 2)  $KpnI$

Need true  $PstI$  control (2 DNA)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Sege W. Khl*

Date

Invented by

Date

Recorded by

*David K. Clarke*

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

Preparation of Competent Yeast Cells.  
TITLE Test Digestions of 3K and 1.5K DNAs.

From Page No. \_\_\_\_\_

Set up YPH 469 250ml culture by inoculating with 2.5ml of  
 YN culture ~ 9am - Will check OD<sub>600</sub> ~ 3pm

Set up the following digestions

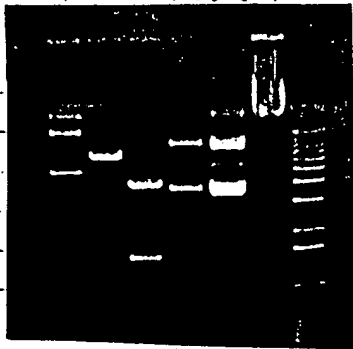
1.5K - Pml  
 1.5K - Sph I  
 3K - Pml  
 3K - Kpn I  
 $\lambda$  - Pml  
 in 20 $\mu$ l - 1ml Enz in each  
 ~ 2hrs at 37°C

Will analyse on a 1% Agarose gel later

Checked Hybridomas - No contamination so far - there are some clumps of  
 cells which hopefully will grow and expand into hybridomas.

clumps - hopefully hybridomas  
 Fibroblasts  
 individual - non-clumped round cells

Will check again in 5-7 days  
 skip 1 sample of negative  
 Pml  $\lambda$  phage DNA



Good; it looks as though Pml  
 is defective, and is not cutting at  
 all either in  $\lambda$  phage or 1.5K and  
 3K clones. Sph I is cutting okay.

Ordered fresh Pml

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Invented by

Date

*David W. White*

Recorded by

*David K. Clarke*

000412

TITLE: \_\_\_\_\_

From Page No. 173

Went through preparation of competent yeast cells *S. cerevisiae* - stored  
ON at 4°C in Li Acetate.

Inoculated 250ml broth (yea) with 5 $\mu$ l, 5 $\mu$ l and 5 $\mu$ l of ON  
yeast + 499 - will check OD<sub>600</sub> tomorrow

Set up ON (0.9K + 3.5K in pCII) in a 250ml broth Amp<sup>r</sup>

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sam W. Kibbe*

Recorded by

*David K. Clarke*

000413

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Preparation of Electrocompetent yeast cells + transformation  
Transformation of LiAc competent cells.  
Maxi prep of H.4K Clone

From Page No. \_\_\_\_\_

Measured  $OD_{600}$  of 3 yeast  $9\text{mL}$  cultures to be used for preparation of electrocompetent cells - Used culture inoculated with  $50\mu\text{L}$  of  $9\text{mL}$  culture - Harvested later and went through transformation protocol for preparation of electrocompetent cells (in the 'Redbook') - Electrotransformed cells with  $100\text{ng}$  and  $1000\text{ng}$  of plasmid - plated out  $1/5$  of total transformants

Also carried out LiAcetate electroporation transformation using  $100\text{ng}$ ,  $500\text{ng}$  and  $1000\text{ng}$  of plasmid - plated out all of transformants

Run through Maxi-plasmid prep with H.4K plasmid - yield appeared to be very low - consistent with the toxic nature of this plasmid - will check on a gel tomorrow

Set up 4  $9\text{mL}$  Mini Yeast cultures in  $3.25\text{mL}$  DOB broth URA<sup>-</sup>

Booked Portugal/Ireland Trip

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne W. K. H.

Recorded by

Daniel K. Clarke

Yeast mini-prep; checking 4.4K maxi preps.  
TITLE Fed Hybrids / O/N 4.4K minis

Project No. \_\_\_\_\_  
Book No. \_\_\_\_\_

175

From Page No. \_\_\_\_\_

Run through mini-prep extractions for Yeast cells. - spin down  
~ 3ml of O/N culture and followed strategy protocol.

Prepared a 1% Agarose gel and loaded ~  $\frac{1}{5}$  of the total prep  
~  $\frac{1}{50}$  of one sample

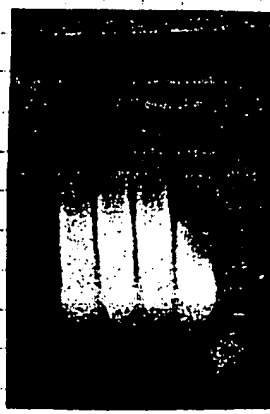
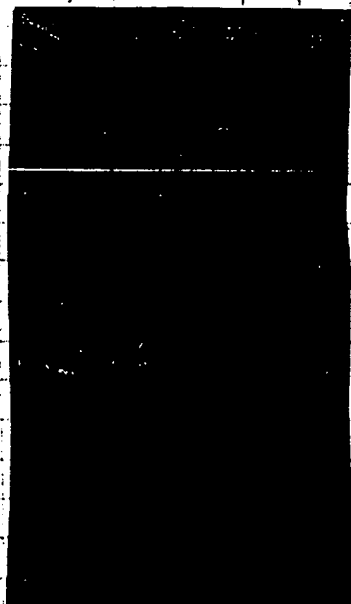
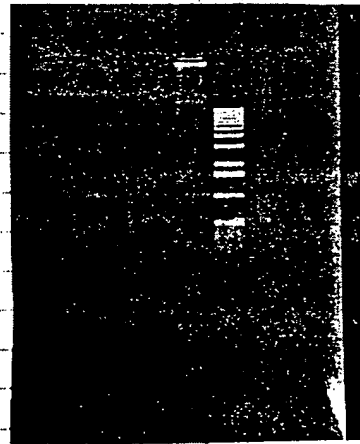
Run 1/2 of Maxi-prep on a 1% gel - doesn't look as though the  
extraction worked - may have to purify the DNA from multiple mini preps  
instead.

4.4K maxi prep.  
1Kb ladder

Removed some of hybridoma  
supernatant from 48 well plates and  
fed with rest of fresh HAT medium.

Set up O/N 4.4K  
Minis (16 in total)

Georges  
Yeast mini prep  
pRS416



Plasmid yields from Yeast look  
very low (or none at all!!)  
Will Run a 2<sup>nd</sup> gel with some  
cell plasmid (pRS416)

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Sege W. Kibbi

Recorded by

David K. Clarke

000415

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Mini preps for 44K.

Check on PstI. One yeast mini prep

TIT

Fre

From Page No. \_\_\_\_\_

Checked hybridomas - will let grow over the weekend, and then possibly plate out into 24 well plates.

checked Yeast transformants - it looks as though there are numerous transformants, with electroporation being most efficient, giving colonies with high of transforming plasmid. Will leave for another few days.

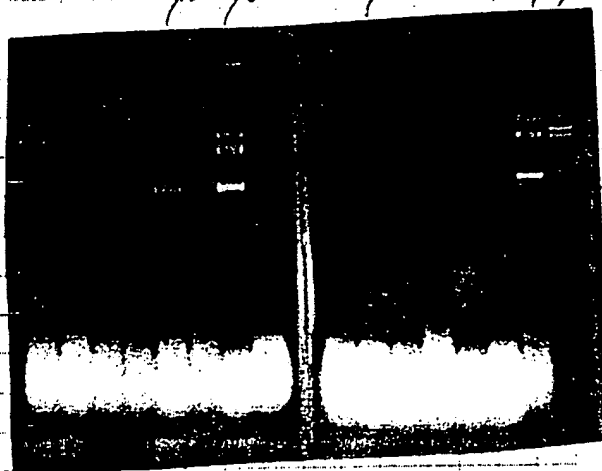
Carried out Mini prep DNA extractions on 44K plasmid X16. - Ran a 10% sample from each prep (sp1)

Set up PstI digestion of  $\lambda$  phage DNA in 20  $\mu$ l at 37°C for ~1 hr. 1 plate

13, 8

5

11

-  $\lambda$  phage DNA - PstI cut

Interestingly, it appears that those on cultures set up from the original plate gave high yields of plasmid, while those set up from later plates contain very little plasmid - the bacteria are dumping down the N° of plasmid copies per cell.

Decided to set up Mini preps from ⑧ and ⑬ mini. Also streaked some cells from ⑧ and ⑬ onto a plate.

Set up 2x 3.5ml on yeast cultures for mini preps tomorrow

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Invented by

Date

Sgt. W. K. Kish

Recorded by

David R. Clarke

000416

TITLE

Yeast mini preps + Restriction analysis.  
Maxi - 4.4K prep

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

177

From Page No. \_\_\_\_\_

Set up Yeast Mini preps x 2 (doubled the amount of Zymolase used in spheroplasting) Lisa went ahead with maxi-prep of ⑧ - potential 4.4K

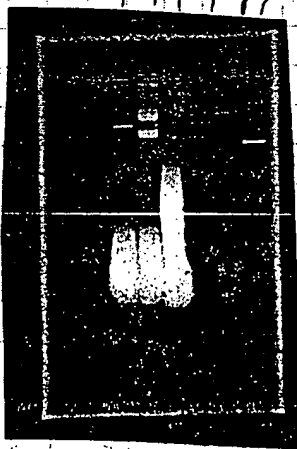
Set up Bam HI digestion on pRS416 + Yeast mini prep - included some RNase A

Prepared a 1% Agarose gel and ran out samples

Lanes / Bam HI cut pRS416 / Bam cut Yeast Mini / Yeast Mini, RNase + / Yeast mini, RNase -

Also ran out mini preps 15/16 from yesterday.

from yesterday: 14 15  
yeast mini - No RNase  
yeast mini - RNase +  
Yeast mini - Bam Cut, RNase +  
Bam Cut pRS416



Seems clear that yeast is not going to be useful to us as there is no sign of the correct sized linear plasmid band.

Will have to consider bundle vines.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Steve W. Kibbe

Recorded by

David K Clarke

000417

From Page No. \_\_\_\_\_

Set up PstI digestion of 3K plasmid (spun down 150 $\mu$ l of Etoh ppt).

Also PstI digested the existing 1.5K material, which has already been digested with SphI/HhaI.

Digestions were done in ~50 $\mu$ l vols with ~3-Spl enzyme for ~3hrs at 37°C.

Possible solutions to cloning problem.

- 1) Colony purify 1.3K and/or 0.9K clones, and then 'cure' the clone(s) of the plasmid by successive passage in non-selective medium.
- 2) Try cloning 1.3K in Baculovirus transfer vector to see if it is stable in that vector system. <sup>transform sure</sup> their host lines.
- 3) Cloning in 2 phases if it will accept 1.5Kb fragments etc.  
A) Will only be useful for in vitro transcription approach.  
(see George).
- 4) Clone in VZV cosmid system. or try cosmid system by itself.  
A) May not be useful if have to clone into cosmid first and go through E.coli.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Steve W. Kubi

Recorded by

David K. Clarke

000418



Gel analysis of *PvuII*/*SphI*/*HhaI* cut 1.5K DNA  
and *PvuII* cut 3K DNA. SAP<sup>+</sup> of 3K DNA (*PvuII* cut).

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

179

From Page No. \_\_\_\_\_

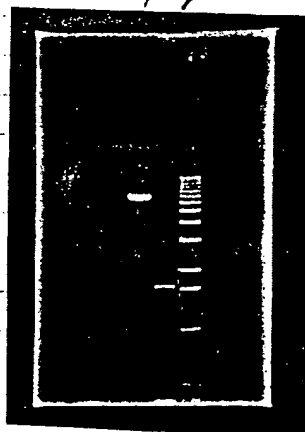
Prepared a 1% agarose gel for analysis of *PvuII* cut 3K and 1.5K DNAs.

Set up phosphatase RXN on *PvuII* cut 3K DNA.

in 100 $\mu$ l final vol - adding 1 $\mu$ l phosphatase - then 3 $\mu$ l after the first half hour.

Checked hybridomas - there are definitely some hybridomas growing as slightly enlarged - round groups of cells ("Pearls").

3K *PvuII* cut.  
1.5K *PvuII*/*SphI*/*HhaI* cut.



The 1.5K clone is still too large by ~100 b.p.

The 3K *PvuII* digest looks okay

Will check 1.5K clone with *EcoRI* and *BstXI*

*EcoRI* should give 924 + 610 bands.  
*BstXI* should give ~1600 bands.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

*Steve W. Lohle*

Recorded by \_\_\_\_\_

*David K. Clarke*

000419

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

Sorting out 'positive' hybridomas.

TITLE Digestion analysis of 1.5K and 3K DNA

Transformation of JC9387 with 1.3K DNA.

From Page No. \_\_\_\_\_

Prepared 200ml L-Agar for JC9387 cloning.

Set up digestions

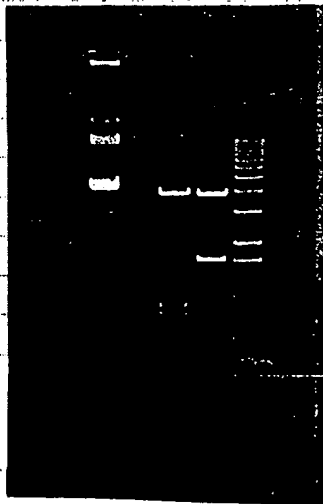
EcoRI cut 1.5K	} in 20µl - 1µl Enz - 2hrs at 37°C.
BstXI cut 1.5K	
KpnI cut 3K DNA (PvuI cut + phosphatased)	} in 80µl
with Spl Enz	

prepared 1% agarose gel for analysis of digests.

Dried down 2µl of 1.3K clone plasmid - resuspended in Spl H<sub>2</sub>O and used

1µl to transform some JC9387 cells.  
 plated out 100, 10, 5µl aliquots  
 of see green transformants.

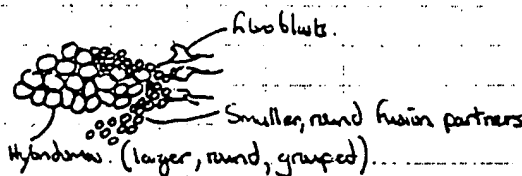
[Will have lisa digest 4.4K  
 plasmid]



EcoRI and BstXI digestions of 1.5K clone indicate that the 1.5K clone is not quite correct by ~ 70-100nt & will set up a fresh RT/PCR rxn on Wed.

The 3K PvuI/KpnI digestion looks fine, but there is not much material remaining (~200ng of the 3K band). Will probably have to repeat these digestions + SAP.

Checked through all six 96 well plates to look for hybridomas, and prepared 2x HAT medium



To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne W. Kibbe

Recorded by

David K. Clarke

000420

From Page No. \_\_\_\_\_

Checked JC 9387 transformants - it does not look as though there are any

white colonies - will leave a few hours more

Also checked streaked out 1.3K clone - there are some single colonies - will pick those later and grow up as mini preps.

Spun down 150  $\mu$ l of 3K clone and PmlI digested in 50  $\mu$ l - 5  $\mu$ l Enz  $\sim$  3 hrs 37°C

Lisa is doing some diagnostic cuts on 4.4K plasmid (EcoRI)

Checked hybridomas - Removed some media and then transferred (with

5-10 up-and-down pipettings) the remaining media too a fresh 46 well plate; refed the original wells and incubated as pairs at 37°C

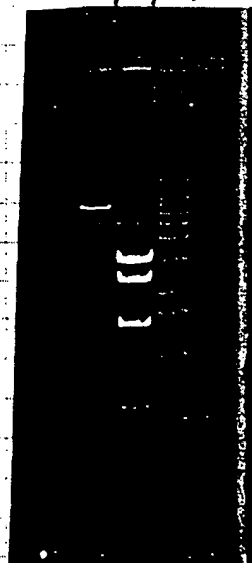
Prepared fresh 14mM Methyl Mercury and 1.85M  $\beta$ -Mercaptoethanol, for RT/PCR RXN.

Set up o/n '3K sure' so Lisa could use to do a Maxi prep later in the week.

also set up eight 1.3K minis from

individual colonies as a prelude to

passaging in the absence of antibiotic



PmlI digest shows  $\sim$  100-200ng of DNA  $\therefore$   $\sim$  2-4  $\mu$ g total

4.4K digest looks good; all 4 predicted insert bands are present adding up to the correct 4.4K size.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Serge W. Klibe*

Recorded by

*David K Clarke*

000421

Project No. \_\_\_\_\_

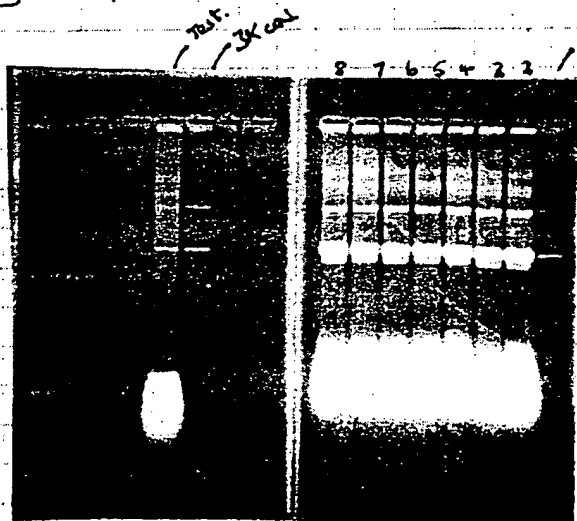
Book No. \_\_\_\_\_

RT/PCR for 1.5K region Sph/Pml.  
TITLE Phosphatasing of PmlI cut SK DNA.

From Page No. \_\_\_\_\_

Carried out phosphatasing of pmlI cut 3K DNA - hot rxn  
in 100µl final vol. with 5µl SAP, 30mins, 37°C then another 2µl SAP, 37°C 30 mins.  
Phenol extracted and EtOH pptd.

Carried out mini preps on 3K mini + 7 x 1.3K minis - ran 2 x 1% agarose  
gels to analyse the products



Will set up a 3K  
maxi prep with 'Test'

3K q/c culture.

Will try to passage all  
seven clones (1.3K)  
in L-B broth minus Amp  
(used Sph inoculation).  
Will check each mini  
prep at 5-passage  
intervals.

Set up RT/PCR rxn in an attempt to recure 1.5K region.

Did a 'Hot-start' for Taq  
Rxn (added Taq at 80°C)

Spun down 0.8ml of genomic DNA  
Dissolved in 3µl H<sub>2</sub>O / 3µl Sph → Pml primer  
Heated to 85°C for 5mins → Ice.

Added 0.6µl 14mM Meth Merc. - 4mins R.Temp

Then added 0.6µl 2-mercaptoethanol - 4mins R.Temp

Added 4µl of this to an RT Rxn in 20µl final vol;  
with 1µl RTase.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Suzanne W. Kibbe*

Date

Invented by

Date

Recorded by

*David K. Clarke*

000422

continued Passage of 1.3K clones without  
Analysis of 1.5K RT/PCR PRODUCTS.  
TITLE Digestion of 3K DNA with KpnI + gel analysis.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

183

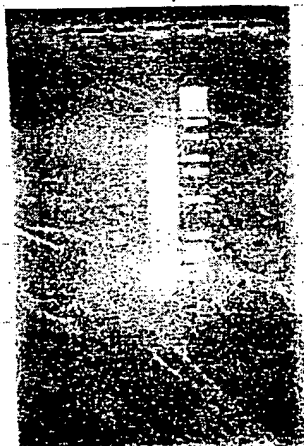
From Page No. \_\_\_\_\_

Prepared 1% agarose for analysis of RT/PCR product. - loaded  
 $\frac{1}{10}$  of total along with 1Kb ladder.

Stored ON 1.3K passages (2-7) w glycerol stocks at  $-70^{\circ}\text{C}$   
(150  $\mu\text{L}$  glycerol + 850  $\mu\text{L}$  ON culture).

Set up KpnI digestion of 3K DNA (PmlI cut + SAP treated).  
In 50  $\mu\text{L}$  final vol.  
Spt Enz. 2-3 hrs. at  $37^{\circ}\text{C}$ .

1.5K RT/PCR products  
1Kb ladder



Tomorrow will run out - Long  
excise the larger DNA band and  
elute the smaller DNA band onto  
NA45 paper

Again, can see a doublet  
in the 1.5 - 1.7K region

This smaller DNA fragment is the  
one that I want

Chloroform extracted and EtOH  
pptd with a couple of  $\mu\text{L}$  of  
glycerol

prepared a fresh batch of NA45 paper.

Ran out a sample of PmlI/KpnI SAP<sup>+</sup> 3K DNA along with a little of the maxi prep  
DNA (Lisai).

Called YEAST expert (Tina Superior) to see if cloning in yeast would be possible;  
He said no.

Prepared some fresh NA45 paper and stored at  $4^{\circ}\text{C}$ .

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Steve W. Kibbe*

Recorded by

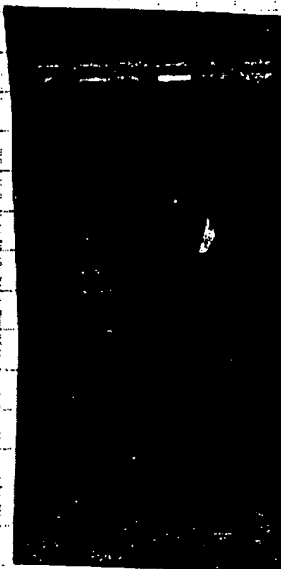
*David K. Clarke*

000423

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE \_\_\_\_\_

From Page No. 183

Good. Gel shows perfectly cut  
3K DNA ~ 30ng in the appropriate band  
20x30 = 600ng of correct DNA  
Will pool with earlier material

The ccc Mazi prep. also looks good.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kline*

Recorded by

*David K. Clarke*

000424

TITLE: Gel purification of 1.5K RT/PCR product.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

185

From Page No. \_\_\_\_\_

Prepared 1% Agarose gel for gel purification of RT/PCR (1.5K) products.

Run bromophenol blue off the bottom to maximise separation of duplex.

Checked hybridomas - difficult to tell if the hybridomas are growing or not -  
Will leave them over the weekend.

Electroeluted 1.5K DNA into NAHS paper (Excised the 1.65K band

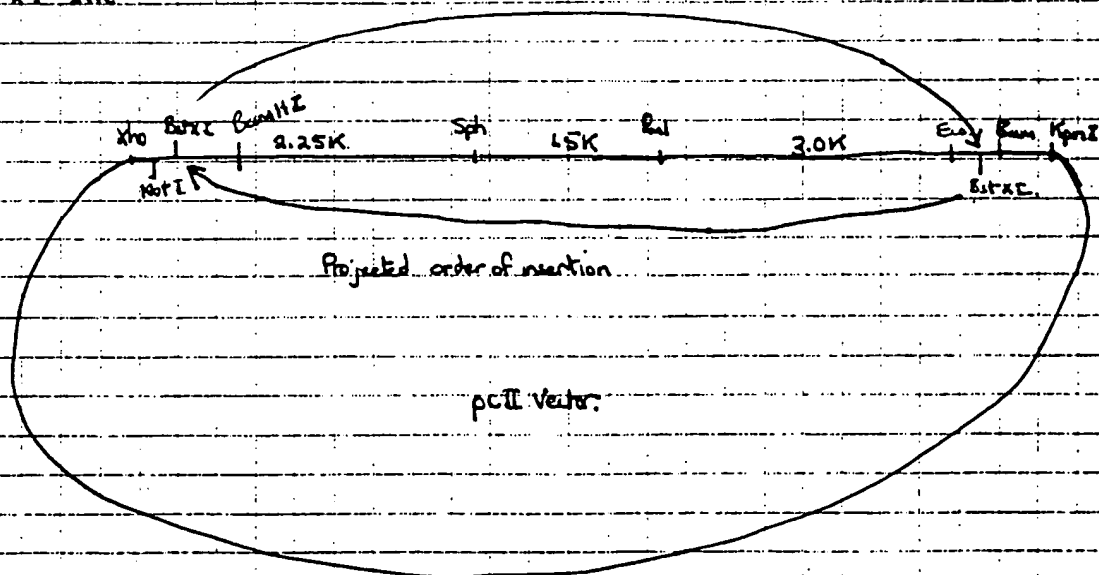
first - before electroeluting) Phenol chloroform extracted and EtOH pptd with

some glycogen.

Lisa continued with the passage of 1.3K clones in the absence of

Antibiotic - I will keep them going over the weekend.

If 1.5K + 3K + 2.25K clone is obtained ( $\approx 6.75K$ ), then may need to flip it around at the  
BstXI site.



To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Sage W. Kibbe*

Recorded by

*Daniel K. Clarke*

000425

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Analysis of 1.5K RT/PCR DNA - Gel purified.  
Passage of 1.3K clones in absence of antibiotic.

From Page No. \_\_\_\_\_

Prepared a 1% agarose gel for analysis of 1.5K gel purified RT/PCR product - Run 1/3 of total purified DNA alongside 1KB marker.

(P4)

Continued 1.3K clone passages 2-8, using a Spl. inoculum into Antibiotic minus L-8.

Gel purified 1.5K RT/PCR product.



Good! gel shows that the 1.5K band is enriched ~10 fold over the 1.65K contaminating band. Will ligate 4pl with ~100ng of pCR II vector o/n Sun → MON.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Steve W. Kibbe*

Recorded by

*David K. Clarke*



QW ligation of gel purified 1.5K RT/PCR product with  
PCR II vector.

TITLE: Passage of 1.5K bacterial clones

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

187

From Page No. \_\_\_\_\_

Took off QW 1.5K cultures and stored a sample of each at  $-70^{\circ}\text{C}$   
in 15% glycerol. - Continued passage with a 5  $\mu\text{l}$  inoculum from each to fresh  
L-8.

Checked hybridomas - it looks as though some of them are growing in the  
sub-culture 96-well plates - will check again tomorrow, and remove a little  
medium, then replace it with HAT+ medium containing O.P.I. (likewise in  
original 96 well plates).

Set up QW ligations at  $12^{\circ}\text{C}$  between purified 1.5K DNA and PCR II vector.

2  $\mu\text{l}$  x 10 buffer  
4  $\mu\text{l}$  of DNA (1.5K ~ 25ng).  
3  $\mu\text{l}$  of Vector ~ 75ng.  
10  $\mu\text{l}$  H<sub>2</sub>O  
1  $\mu\text{l}$  Enz  
20  $\mu\text{l}$

set up a control minus  
1.5K DNA.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Seay A. Kibler*

Recorded by

*David K. Clarke*

000427

From Page No. \_\_\_\_\_

Prepared L-agar (200ml) for transformations, to be done later today.

Lisa went ahead with mini-preps and gel analysis of 1.3K clones.

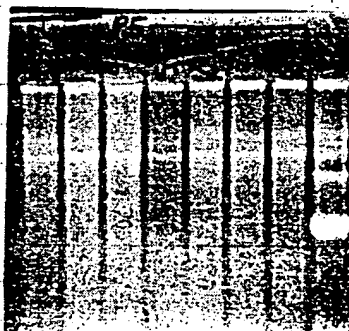
— prepared RPMI medium + O.P.I. (60ml) for growth of hybridomas

Removed some of old medium and replaced with 100µl of O.P.I. medium.

Transformed 100µl of 'SURE' cells with Spl. of o/n ligation mixture —  
heat shocked at 42°C for 45secs. Incubated in 90µl of SOC for 1hr to

allow phenotypic expression. (with frequent shaking). Prepared IPTG/X-Gal Amp<sup>r</sup> Agar  
plates.

Sp. Plated out 3x20µl on  
Amp<sup>r</sup> plates, and 1x20µl for  
CON. → o/n 37°C



It would appear that virtually  
all of the plasmid has been  
lost after 5 passages —  
although there are some traces  
in 2, 3, 6, 7, 8.

- 1) Will proceed with population passages through P<sub>10</sub>.
- 2) Will check back on P<sub>1</sub> → P<sub>2</sub>, P<sub>2</sub> → P<sub>3</sub> and P<sub>3</sub> → P<sub>4</sub> (mini-preps).
- 3) If little plasmid is present on some of the earlier passages — will streak out to single colonies — then check them on ~~amp~~ amp<sup>r</sup>/amp<sup>r</sup> replicate plates then recheck the amp<sup>r</sup> colonies for absence of plasmid.
- 4) Then prepare competent cells from amp<sup>r</sup> clone(s) and transform with 1.3K DNA — if stable transformants are generated then the exp. is successful!

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Invented by

Date

*Serge W. Khle*

Recorded by

*David K. Clarke.*

000428

9m Minus/master of potential 1.5K clones.

Project No. \_\_\_\_\_

TITLE Mini prep analysis of 1.3K clones (for loss of plasmid)

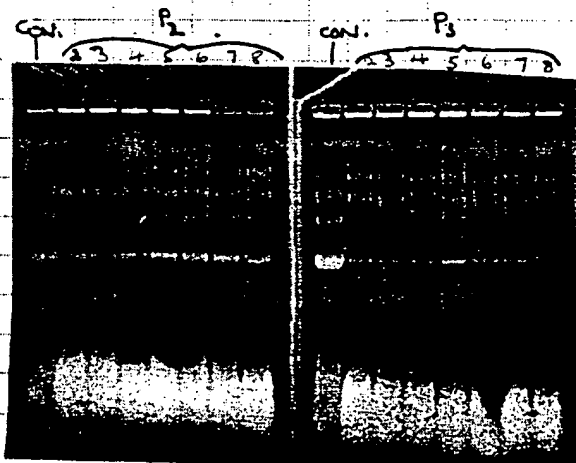
Book No. \_\_\_\_\_

189

From Page No. \_\_\_\_\_

Checked transformants - there are numerous white colonies on plates where 1.5K DNA was ligated into pCR II vector. Control plate shows only blue colonies - seems like there should be some transformants - will streak out ~ 36 and set up 10 9m mini preps.

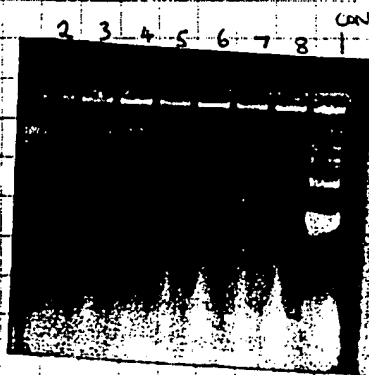
Prepared 3x 1% agarose for analysis of mini preps  $P_2 \rightarrow P_3 \rightarrow P_4$  (to find where 1.3K plasmid is first lost from bacterial clones).



Set up 9m minis for potential 1.5K clones.

There is a clear gradient of plasmid loss from  $P_2$  through  $P_4$  and  $P_5$ .

Should be able to streak out  $P_4$  colonies on Amp<sup>r</sup> LB agar (for 2-7), then look at 3-4 colonies from each by mini prep - after replicate plating on Amp<sup>r</sup> plates.



To Page No. \_\_\_\_\_

Witnessed & Understood by me.

Date

Invented by

Date

*Steve W. Koble*

Recorded by

*David K. Clarke*

000429

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE \_\_\_\_\_

Potential 1.5K ~~maxi~~ clone mini preps and digestion  
+ gel analysis. Growth of potential 1.3K plasmid minus clones.

From Page No. \_\_\_\_\_

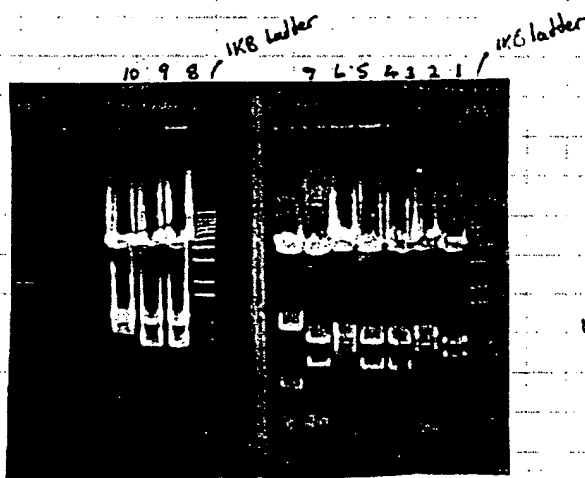
TIT

Fro

Carried out 10 mini preps on potential 1.5K clones - set up

EcoRI digests in 20 $\mu$ l with 1 $\mu$ l Enz + RNase for ~2hrs at 37°C.

prepared 2 x 1% agarose gels for analysis of digestion products.

Prepared 1 litre of L-Agar for streaking out potential 'plasmid-minus' bacteria  
(1.3K clones 2-7). Streaked each of these clones out to single  
colonies (from P<sub>4</sub> populations).3, 8, and 9 also look  
as though they contain  
inserts of the correct  
size. Will check  
maxis with BstXI, and  
EcoRI cuts.

Selected clones 4 and

6 to be grown up as

Maxi preps in 250ml O<sub>2</sub>N

cultures.

Streaked out 1.3K clones 2-7 to single colonies - will replicate  
plate 10 colonies from each clone on Amp<sup>r</sup> plates to check for loss of  
the 1.3K plasmid.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Invented by

Date

Serge W. Kibbe

Recorded by

David K. Clarke

000430

Replicate plates of potential 1.3K clones  
Maxi preps of 1.5K clones (4) and (6) and EcoRI/BstXI  
TITLE Digestion analysis of these clones.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

191

From Page No. \_\_\_\_\_

Checked streaked out 1.3K clones - will pick up to 10 clones from each plate for Amp<sup>r</sup>- selection.

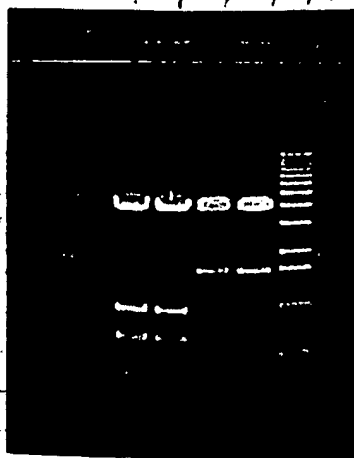
Meanwhile checked all 6 48-well plates for evidence of hybridoma cell growth. - there are about 40-50 hybridomas in total - will check them again on SAT. or SUN. and sub-culture into 24 well plates

Streaked out 1.3K clones and grew on at 37°C.

Lisa carried out maxi preps on clones (4) and (6) for 1.5K region - EcoRI and BstXI digestions on both (4) and (6) were set up in 20 µl of final vol - used 1 µl of DNA, 1 µl of Enz - 1.5 hrs at 37° and 55°C respectively.

Prepared 1% agarose gel for analysis of digestion products.

Lisa continued with passage of 1.3K clones to get rid of plasmid.



Good! Looks like the 1.5K clones are both correct.

The BstXI frag should be 1,573 (and is).

The EcoRI frags. also look to be the correct size.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Steve W. Khr*

Recorded by

*David K. Clarke*

0000431

Project No. \_\_\_\_\_

Bulk digestion of 1.5K plasmid (4)

Book No. \_\_\_\_\_

TITLE

SphI and PstI.

From Page No. \_\_\_\_\_

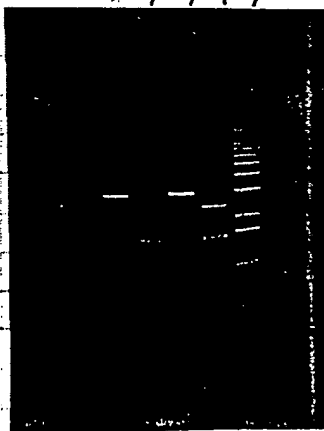
checked growth of replicate plate '1.5K' clones - Good! it appears that all the clones are Amp sensitive which would indicate they have lost their plasmid.

Set up SphI digestion on 1.5K DNA. - used 5 $\mu$ l of DNA ~ 5 $\mu$ g in a 50 $\mu$ l digest with SphI enzyme ~ 2hrs at 37°C.

Also set up XhoI digestion of 3 $\mu$ g of pET DNA in 50 $\mu$ l with 2 $\mu$ l Enz - 2hrs at 37°C.

Phenol chloroform extracted and EtOH pptd. Then set up PstI digestion of SphI digested 1.5K and KpnI digestion of XhoI digested pET DNA.

Retained small samples from each digestion for gel analysis.



Prepared 1 litre of fresh Hybondma medium containing O.P.I. but minus HAT (to be added separately later).

lisa filter sterilized

Good! looks as though double digests have worked. Will need to do a final XhoI digestion on 1.5K DNA to remove Vector DNA from the 1.5K band.

No. 4 (1.5K).

BamHI SphI

PstI XhoI SphI

SphI pos. 368... in Vector:  
NotI " 353

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Serge W. Kiehl*

recorded by

*David K. Clarke*

000432

Title. HpaI digestion of 1.5K DNA  
Phosphatasing of pCII DNA.  
Gel purification of 2.25 and 3K DNAs.

Set up HpaI digestion on 1.5K DNA to remove contaminating Vector DNA. - in 50 $\mu$ l final vol - 5 $\mu$ l Enz ~ 2-3hrs at 37°C.

Set up Phosphatase rxn on Xho/KpnI digested pCII vector DNA - in 30 $\mu$ l with 2 $\mu$ l of SAP, 30mins - then 1 $\mu$ l more SAP. Will freeze both until tomorrow, and then gel purify.

Prepared a 1% agarose gel, and ran out 2.25K DNA and 3.0K DNA for gel purification onto N445 paper.

Checked hybridomas, and 'eumarked' those to be subcultured into 24-well plates tomorrow. Also found a few 'new' hybridomas.

Completed electroelution and phenol/chloroform extracted; then precipitated the DNA with a little glycogen.

Steve W. Kibbe

Recorded/Invented by:

David K. Clarke.

Date:

0000433

Title.

Prepared a 1% agarose gel for Electroelution of pET DNA (srp<sup>+</sup> etc) and 1.5K DNA (SphI-PstI-HaeI cut).

Sub-cultured hybridoma cells into 24 well plates (23 in total) - those that looked well-grown only - pipetted up and down fairly vigorously, then transferred to the 24 well plate (containing 1ml growth medium OPZ<sup>+</sup>, HAT<sup>+</sup>). Proceeded then to stain agarose gel, and continued with electroelution onto N445 paper - phenol extraction and precipitation with a small quantity of glycogen.

Set up o/w cultures of 'cured' 1.3K clones - Set up 4 minis (all N<sup>o</sup>: 4) from series 2, 3, 4, 5, - will make competent cells from each of 10 tomorrow.

Serge W. Khle

Recorded/Invented by:-

David K. Clarke

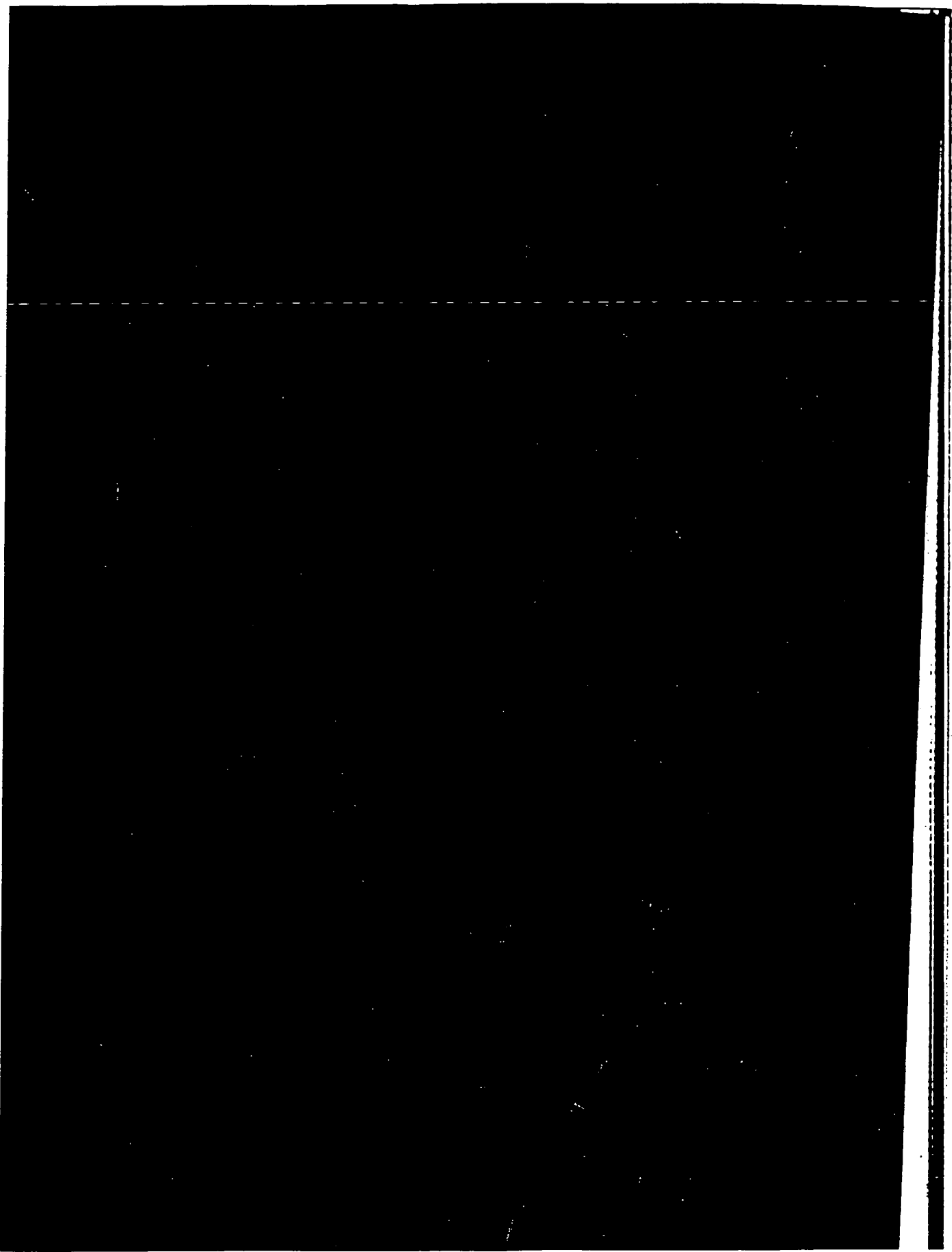
Date:-



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■000436

BOOK 3

LABORATORY  
NOTEBOOK

AVIRON

DAVID CLARKE

1000437

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000438

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0000439

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  - Use ink for permanence -- avoid pencil.
  - Record your work as you progress, including any spur-of-the-moment ideas which may be developed later.
  - Avoid making notes on loose paper to be recopied.
  - Record your work in such a manner that a co-worker can continue from where you stop. You might be ill and to protect your priority it could be urgent that the work continue while you are absent.
3. • Give a complete account of your experiments and the results, both positive and negative, including your observations.
  - Record all diagrams, layouts, plans, procedures, new ideas, or anything pertinent to your work including the details of any discussions with suppliers, or other people outside the Company.
  - Do not try to erase any incorrect entries; draw lines deleting them, note the corrections, sign and date the changes. This extra care is worthwhile because of the necessity of original data to prove priority of new discoveries.
4. • After entering your data, sign and date the entries.
  - Explain your work to at least two witnesses who are not co-inventors, and have them sign and date the pages in the place provided.
  - Record the names of operators and witnesses present during any demonstration and have at least two witnesses sign the page. If no witnesses are present during an experiment of importance, repeat it in the presence of two witnesses.
5. Since computer programs can be patented these instructions apply to the development of computer software. In this case a description of the structure and operation of the program should be recorded in the notebook, together with a basic flow diagram which illustrates the essential features of the program. In the course of developing the code, the number of lines of code written each day should be recorded in the notebook, together with a statement of the portion of the flow diagram to which the section of code is directed.
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Preparation of Competent cells 2-5  
TITLE Gel analysis of NA45 purified 3K, 2.25K, 1.5K and pCII DNAs

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

1

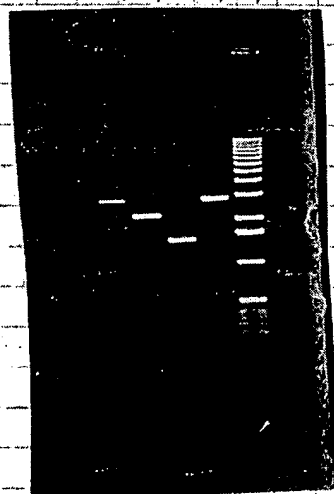
From Page No. \_\_\_\_\_

Inoculated 100ml L-B broth with 0.5ml of Oxy culture  
(from Oxy minis of 2, 3, 4, 5 - 'cured' 1.3K clones) - to prepare  
competent cells

Meanwhile prepared 300ml L-Agar, Amp<sup>+</sup> for plating out transformants  
Spin down gel purified 3K, 2.25K, and 1.5K + pCII DNA and resuspended  
in 20µl H<sub>2</sub>O - then loaded 4µl (1/5) for gel analysis...

3.0K  
2.25K  
1.5K  
pCII vector  
1KB ladder

Will use 4µl of pCII  
Vector + 4µl 3K, and 2µl  
2.25K - in 20µl ligat<sup>n</sup> vol.  
1µl Enz, and will sample at  
10 mins, 30 mins and 1hr



Will attempt to ligate pCII vector  
(2.9K) with 3K and 2.25K fragments

pCII DNA is SAP<sup>+</sup> Kpn/Xho

2.25K is XhoI cut, and SphI  
cut with SphI site SAP<sup>+</sup>

3K is PstI cut with SAP<sup>+</sup> at that site  
and KpnI cut

Continued with transformations - Dried down 3µl of 1.3K DNA and resuspended in 20µl  
H<sub>2</sub>O - used 1µl to transform 2, 3, 4, 5, competent cells - plated out 200µl of  
1ml final vol. (after 'rescue' of cells) onto X-gal/10% Amp<sup>+</sup> Agar.

Also set up Oxy minis on 1.5K 3, 4, 6, 8, 9 to recheck and freeze down 1.5K  
clones.

Checked hybridoma cells - the best mostly good - will refreeze older cells tomorrow  
and

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

Steve W. Kibbe

Recorded by

David K. Clarke

0000457

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

Trial ligation of 3K and 2.25K into pCII vector  
TITLE on minis of potential 1.3K transformants

From Page No. \_\_\_\_\_

Checked bacterial transformants - two of the four transformations appear to have worked - with a small no. of white colonies visible - the other two plates do not appear to have any colonies - but will incubate all four for a few hours more.

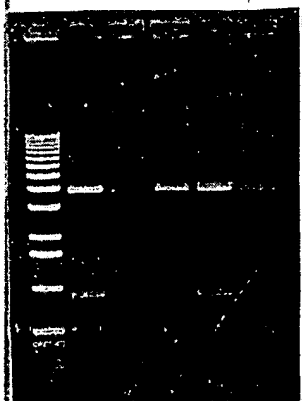
Set up pCII, 3K, and 2.25K ligation

Prepared a 1% agarose gel for analysis of ligation products.

Lisa did mini preps on potential 1.5K clones.

4 $\mu$ l 3K DNA2 $\mu$ l 2.25K DNA4 $\mu$ l pCII DNA1 $\mu$ l T<sub>4</sub> ligase2 $\mu$ l x10 buffer7 $\mu$ l H<sub>2</sub>O2 $\mu$ l • 37°C for 10mins, 30mins, 1hr

Fed older hybridoma cells with from fresh O.P.E. + HAT medium.



Lisa's mini preps of 1.5K clones - to be frozen down as glycerol stocks



None of these bands are of the correct size to be ~8K required. Will proceed with double ligation i.e. 2.25K + pCII DNA (2 $\mu$ l each in 2 $\mu$ l final vol.)

It may be better to put clones together one at a time i.e. get a 1.5K clone

in suitable orientation and add it onto the 3K clone - then add the 2.25K. Should be able to flip the 1.5K DNA with BstXI digestion + religation.

Set up o/n mini preps of 8 potential 1.3K clones from 'LATE (5)' cells:

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Steve W. Kuhl

Recorded by

David K. Clatke.



Mini prep analysis of 1.3K clones from 'LATE S'

Project No. \_\_\_\_\_

TITLE Ligation of pCII DNA + 2.25K DNA

Book No. \_\_\_\_\_

3

From Page No. \_\_\_\_\_

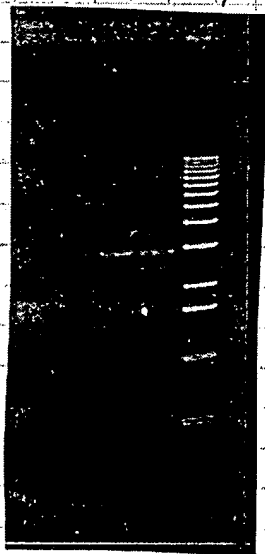
<sup>8</sup>  
Run through mini-preps of potential 1.5K clones from 'LATE S'

Prepared 1% agarose gels for analysis of EcoRI digests of mini prep DNAs.

Lisa carried out mini-preps on 1.5K clones to determine orientation of insert.

Set up a 2-way ligation between pCII DNA and the 2.25K clone

1hr, 30min, 10min, 1Kb ladder



2µl 2.25K DNA

2µl pCII DNA

2µl x10 RXN buffer

13µl H<sub>2</sub>O

1µl Enz

29µl

Sampled at 10mins, 30mins, 1hr at 37°C

Loaded/run gel for analysis

1.3K clones look good

XbaI analysis of 1.5K clones



ccc phim

There are no clear candidates for the 5.25K expected band.  
May abandon this approach.

1051 + MCS 6ps = ~ 1100bp

⊗ looks like the one I want based on partial digestion products. - Will set up an ON/culture tonight to check

by third day tomorrow

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Shige W. Khle*

Date

Invented by

Date

Recorded by

*David K. Clarke*

0000459

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

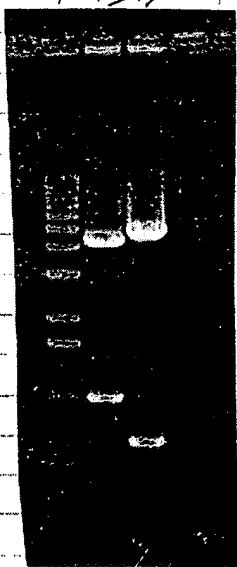
Bulk digestion of 1.3K clone, to provide insert + vector (EcoRI), and gel purification.  
Determination of orientation of 1.5K clones

From Page No. \_\_\_\_\_

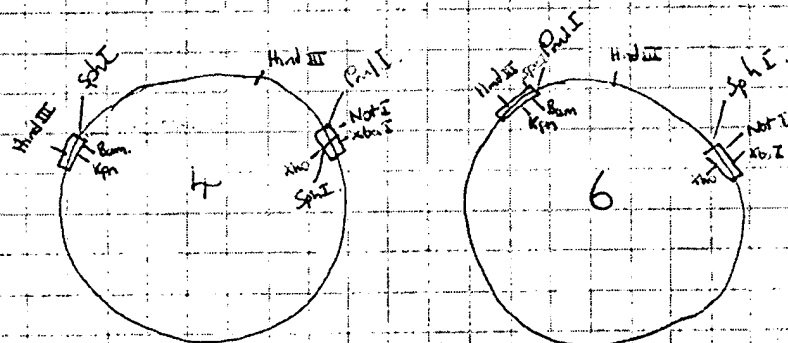
Lisa is doing Hind III digestion on (4) + (6) 1.5K clones to do a final determination of orientation.

Set up bulk digestion of 1.3K to prepare insert + vector to flip the 1.3K insert around in the EcoRI site. Can use a BstXI digestion to determine the orientation of the resulting clones.

1.5K clones (Hind III cut)



Good, it appears that (4) and (6) are in opposite orientations.



Clone Orientations.

Completed electroelution of 1.3K DNA (vector + insert) - Phenol Chloroform extracted and EtOH pptd with 2 $\mu$ l of glycogen.

Set up q/n 'LATES' - to freeze down glycerol stock.

Checked hybridomas - they appear to be growing fairly well for the most part; Will decide how to proceed tomorrow.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sage W. Khali

Recorded by

David K. Clarke

Gel analysis of purified 1.3K and Vector DNAs.  
Restraining of clone banks.  
TITLE Large scale digestions of 1.5K/2.25K with HhaI.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

5

From Page No. \_\_\_\_\_

Prepared 500ml L-Agar (Amp<sup>r</sup>) to restreak clone banks.

Spun down gel purified '1.3K and Vector' DNA and resuspended in 8µl H<sub>2</sub>O. -

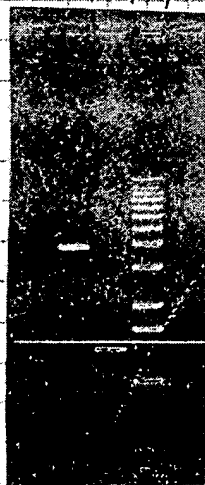
analysed 2µl of each on a gel, along with 1KB ladder.

10.30am - set up 'large-scale' digestions of 2.25K and 1.5K DNA with HhaI -

Used 6µl of enzyme in 60µl final vol for 4-6hrs. Then phenol/chloroform  
extracted and EtOH pptd.

Runed Agar plates - Dried.

Checked Hybridomas - Sub-  
cultured 3 more hybridomas  
into 24 well plates - checked  
those already growing - will  
collect ~50µl of supernatant  
from the most densely populated plates  
tomorrow, and sub-culture into  
25cm<sup>2</sup> Flasks.



Good - both the insert and Vector have been  
purified in reasonable amounts.

Will proceed with phosphorylation of  
Vector component. (~400ng).

Set up phosphatase rxn  
on Vector component in 20µl,  
using 1µl Enzyme, followed by a  
2<sup>nd</sup> 1µl aliquot after ~30mins at  
37°C - proceeded to phenol/chloroform  
extract and EtOH ppt.

To Page No. \_\_\_\_\_

Witnessed & Understood by me.

Date

Invented by

Date

*Steve W. Kille*

Recorded by

*David K. Clarke*

0000461

From Page No. \_\_\_\_\_

[Thought<sup>d</sup> consider subcloning 2.25/1.5 into pCI Vector]

Set up SphI digestions on 2.25K and 1.5K DNAs (HhaI digested)

Did Rnase in 60 $\mu$ l final vol. - used bph enzyme for ~3hrs at 37°C; then phenol chloroform extracted and EtOH ppt.

Collected hybridoma supernatant from most densely populated 24 well plates, before sub-culturing into 25cm<sup>2</sup> bottles. (Collected 500 $\mu$ l from each hybridoma - six in all were sub-cultured today)

Removed Bacterial master plates from 37°C and stored at 4°C - all had grown adequately.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Serge W. Khl

Recorded by

David K. Clarke

**TITLE**

Book No. \_\_\_\_\_

7

Set up digestions of 2.25K DNA and 1.5K DNA. Each in 50 $\mu$ l final vol. with XhoI for 2.25K and SpeI for 1.5K. Used Spt. Enzyme in each case. Set up a control for SpeI (ordered Sigma new SpeI).

Prepared a 1% agarose gel for final analysis of digests - will make a 2nd gel purification of fragments later today.

Fed hybridomas in 96well plates in the hopes of coaxing them to grow out a little more.

cut away from  
the top of the  
plate

spit out per well (con) — Enzyme appears to be working at a reasonable efficiency

1 kb ladder

$50 \times 100 \text{ ng} = 5 \mu\text{g}$  of 1.5K DNA  
 $50 \times 50 = 2.5 \mu\text{g} = 3 \mu\text{g}$  of 2.25K DNA

Completed Gel purification of DNA bands.  
Phenol/chloroform extracted and EtOH pptd

Date \_\_\_\_\_

**To Page No.**

Recorded by

Recorded by David K. Clarke

000463

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

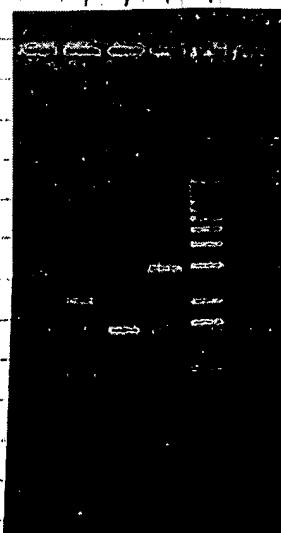
TITLE

Analysis of gel purified 1.5K, 2.25K DNA  
Transformation of 'Late S' with (1.3K+vector)  
Phosphatizing of 1.5K DNA

8

From Page No. \_\_\_\_\_

Inoculated 100ml L-B with 0.5ml ON 'LATE S' - for competent cell production

Prepared a 1% agarose gel for analysis of gel purified 1.5K and 2.25K gel purified DNAs -  
Will look at  $\frac{1}{2}$  pt -  $\frac{1}{4}$  of total. Will also look at XhoI digested pCDNA - 1  $\mu$ l of 3  $\mu$ lPrepared 500ml L-Agar for plates to be used in transformation. - added Amp - poured  
- dried. Did transformation using 1  $\mu$ l of ligation mixture (of 20  $\mu$ l). Heat-shocked 42°C  
for 45 sec, rescued for 1 hr and plated out on X-gal<sup>+</sup>/IPTG<sup>+</sup> plates.Carried out phosphatizing of XhoI cut pCDNA II - in 20  $\mu$ l final vol, - used 2  $\times$  1  $\mu$ l  
of enzyme in a 1 hr total incubation.  
Phenol extracted and EtOH pptd.Carried out sub-culture of hybridoma cells  
into 25cm<sup>2</sup> flasks - stored 500  $\mu$ l of each  
supernatant for assay in neutralization assay.Gel to analyse amount  
of DNA obtained from  
gel purification.There appears to be  
 $\sim 100$  ng  $\times 40 = 4 \mu$ g of  
1.5K DNA.  
 $\sim 2 \mu$ g of 2.25K DNA.Set up phosphatase rxn on all of 1.5K  
DNA in 5  $\mu$ l final vol.5  $\mu$ l  $\times 10$ 40  $\mu$ l containing DNA3  $\mu$ l Enz + 2  $\mu$ l Enz after $\sim 30$  min - then phenol/chloro  
extracted and EtOH pptd.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Amp W. Kuhl

Date

Invented by

Date

Recorded by

Wendy K. Clasher

000464

From Page No. \_\_\_\_\_

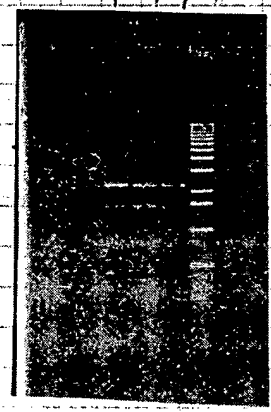
set up Spe I digestion of Xho I cut/SAP<sup>+</sup> pCII DNA.  
 In 30µl final vol - with 3µl Enzyme.  
 Prepared a 1% agarose gel for gel purification of pCII DNA.

Proceeded to Electrophore and phenol extract the Spe I/Xho I/SAP<sup>+</sup> pCII DNA.  
 EtOH ppted with 1µl of glycogen.

Meanwhile set up ligation between 1.5K and 2.25K DNA

hr. 30 min. 10 min. 15 min. 1 hr. 2 hr.

Can see a  
 very low level  
 of 3.75K  
 product, which  
 I require



- 2µl 1.5K DNA
- 2µl 2.25K DNA
- 3µl x10 Xho I buffer
- 3µl x10 BSA
- 3µl 10MM ATP
- 2µl Enz (Xho I)
- 1µl DNA Ligase
- 14µl H<sub>2</sub>O
- 30µl

Will repeat this ligation tomorrow  
 but use double the amount of each  
 DNA used, and leave O/N at  
 37°C. - will then add a little extra  
 Xho I the following morning.

Checked the hybridoma cells - they are all doing  
 okay - removed 500µl of medium from the remaining 24  
 well plates and added 500µl of fresh medium. Will subculture  
 96well plates into 24well plates tomorrow.

Set up a scheme for Lisa to carry out a "back-up cloning" procedure for the addition  
 of the 1.5K to the 2.25K clone.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

*Serge W. Kuhl*

Recorded by \_\_\_\_\_

*David K Clarke*

0000465

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE \_\_\_\_\_

Mini preps + digestion + gel analysis of potential 1.3K clones  
Ligation of 1.5K + 2.25K <sup>BstXI</sup> in  
Subculture of hybridomas

From Page No. \_\_\_\_\_

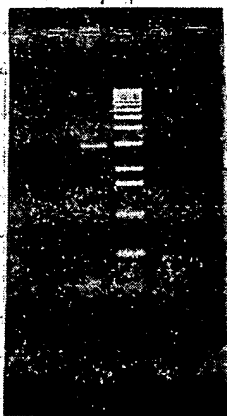
Carried out mini prep DNA extractions on potential 1.3K clones - and then set up *Bst* XI digestions, in an attempt to diagnose clones in either orientation; (however *Bst* XI is not a good choice - so repeated extractions and digested with *Bst* I.)

Also ran out Gel purified *peI* DNA (*Xho*I/*Spe*I cut and partially SAP'd) to check amount of DNA

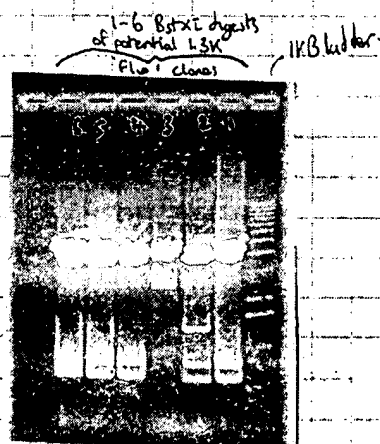
proceeded to set up ligation between 2.25K DNA and 1.5K DNA in the presence of *Xho* I.

4  $\mu$ l 1.5K DNA  
4  $\mu$ l 2.25K DNA  
3  $\mu$ l  $\times 10$  *Xho*I buff.  
3  $\mu$ l  $\times 10$  BSA  
3  $\mu$ l DNA ATP  
2  $\mu$ l *Xho* I.  
1  $\mu$ l DNA lig.  
10  $\mu$ l H<sub>2</sub>O

30  $\mu$ l ON 37°C.



There is ~ 30ng/ $\mu$ l - total  
20  $\times$  30 = 600ng of DNA.  
remaining



Carried out a 2<sup>nd</sup> mini prep extraction and set up *Bst* I digests.

N<sup>o</sup> 2 looks like a head-to-tail dimer  
N<sup>o</sup> 3 looks like recombined vector

To Page No. 11

Witnessed &amp; Understood by me, \_\_\_\_\_

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Steve W. Kibbe

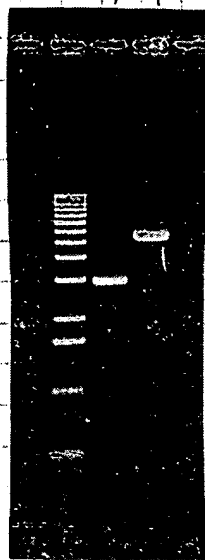
Recorded by \_\_\_\_\_

David K. Clarke

000466



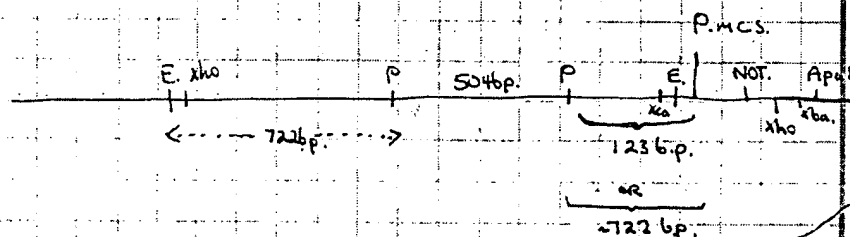
From Page No. 10



Lisa's *Apa*I digest of  
p.c.II DNA and (+) 1.5K DNA.  
Both appeared to work fairly well.

Sub-cultured hybridomas in  
96-well plates into 24-well  
plates (11 in total) - Pipetted  
fairly vigorously and transferred into  
1ml fresh O.P.E. medium + HAT.

*Pst*I digestion of 1.3K gives a 504bp. internal  
(in 1.5K) fragment.



Set up own master plate of 1.3K clones

Witnessed &amp; Understood by me.

*Steve W. Kuhl*

Date

Invented by

Recorded by

*David K. Clarke*

Date

To Page No. \_\_\_\_\_

000467.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE \_\_\_\_\_

Analysis of Pst I digests of potential 1.3K 'Flip' clones  
Analysis of 1.5 + 2.25K ligation/Xho I runs.

Ligation of 1.5K + 2.25K into pII DNA vector

From Page No. \_\_\_\_\_

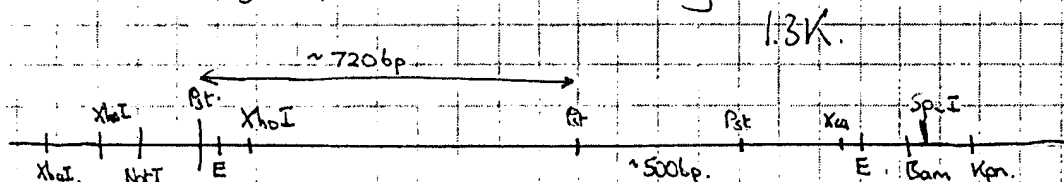
Ran out all six potential 1.3K clones - Pst I digested - also ran out  
6 $\mu$ l ( $\frac{1}{5}$ ) of 2.25/1.5K ligation/Xho mixture to check ligation pattern.

There are some high m.w.  
DNAs generated by the 1.5K +  
2.25K ligation - Phenol/chloro  
extracted the remaining RNA  
and set up a ligation with  
some pII vector (Xho I -  
Spe I cut).



All of the 1.3K containing clones are  
in the same approximate orientation  
(even the one containing the head to  
tail insert).

The Pst I digest pattern indicates the following orientation within the m.c.s.



Page 161 of BK 2.  
May be able to cut 4.4K with Xho I and Xba, then purify  
the large DNA, and ligate to Spe I/Xho I cut (1.3K). Spe I is compatible  
(will get live to grow up more 1.3K DNA.) with Xba I

Will also need to prepare electro competent 'late S' cells.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Suzanne W. Khle

Recorded by

David K. Cluskey

From Page No. 12.

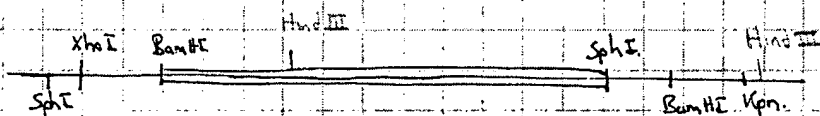
Set up an o/n ligation between ligated 1.5K and 2.25K into XhoI/SpeI —

— Digested pCII DNA.

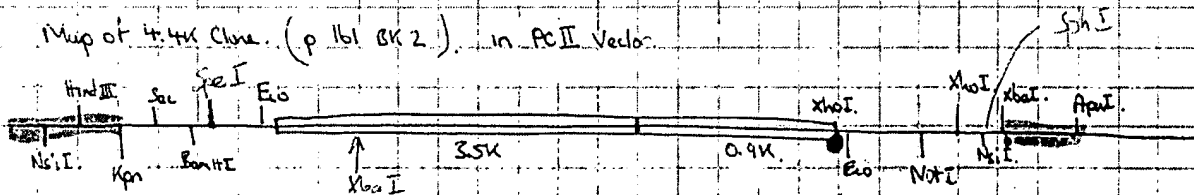
Have 10-20ng total of correct sized DNA band will ligate with  
~ 200ng of pCII vector.10  $\mu$ l H<sub>2</sub>O7  $\mu$ l pCII DNA2  $\mu$ l X101  $\mu$ l Enz20  $\mu$ l 37°C o/n.Prepared Amp<sup>r</sup> Agar plates for transformations tomorrow. — dried and stored at 4°C.

Checked hybridoma cells

Map for 2.25K clone (p168 BK 2).



Map of 4.4K clone (p161 BK 2) in pCII Vector.

Subcultured hybridomas (R5V) 1-6 into 25cm<sup>2</sup> Flasks in a 1-2 splitHybridomas ⑧, ⑩ and ⑪ look good for freezing — will freeze down tomorrow if they  
shl look good.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne W. Kohle

Recorded by

David K. Usher

0000469

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Transformation of 'SURE' cells with 1.5K + 2.25K in  
 pCII. Digestion of 4.4K + 1.3K.  
 Hybridomas 7-13 frozen

From Page No. \_\_\_\_\_

Set up restriction digest of 4.4K DNA - XbaI.

5  $\mu$ l of DNA in a 50  $\mu$ l Rxn, with 4  $\mu$ l enzyme for ~3 hrs at 37°C.

Also set up SpeI digest of 1.3K DNA (50  $\mu$ l spin down from above) in 50  $\mu$ l with 4  $\mu$ l of enzyme for ~3 hrs at 37°C

Then phenol extracted and EtOH ppt<sup>ed</sup>. Saved  $\frac{1}{50}$  each for gel analysis

Carried out transformation of 'SURE' cells with ligated 1.5K and 2.25K into pCII vector - prepared X-gal / IPTG / Amp<sup>r</sup> plates.

Checked Hybridomas - will freeze down 7 through 13 today and probably do 1-6 tomorrow. Will also do some more sub-culturing tomorrow.

Used 'freezing medium' according to Kathy's protocol (+Gln)

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Steve W. Kuhl

Recorded by

David K. Clarke

0000470

ON Minis of potential 1.5K + 2.25K in PCR.  
XhoI digestion of 4.4K + 1.3K DNAs.  
Hybridoma subculture/Freezing.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

15

From Page No. \_\_\_\_\_

checked 1.5K + 2.25K in PCR transformants - there are quite a few whites  
- Will set up ON minis + Master -

Will cut with ~~EcoRI~~ Hind III.

Set up XhoI digestion of 1.3K DNA in 30 $\mu$ l with 2 $\mu$ l of enzyme for ~4hrs at 37 $^{\circ}$ C  
also set up XhoI digestion of 4.4K with 5 $\mu$ l of enzyme for ~4hrs at 37 $^{\circ}$ C.

Will Phenol extract and EtOH ppt 4.4K DNA prior to phosphatasing.  
Will freeze 1.3 K. (removing 1 $\mu$ l for gel analysis) prior to gel purification.

Sub-cultured remaining hybridomas in 96 well plates into 24 well plates;  
also subcultured hybridomas 14 through 26 into 25cm<sup>2</sup> Flasks, from 24 well  
plates - saved 500 $\mu$ l of supernatant from each for assay purposes.

Also froze down two more hybridomas from 25cm<sup>2</sup> Flask (1 + 6)

Set up ON 1.3K culture for Lisa to use as inoculum for Maxi prep.

Also set up a large batch of mini preps to check for 1.5K + 2.25K clone  
34 in total.

Selected on Riv paper for Ying to cover in RA Journal club.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Serge W. Kihl*

Recorded by

*David K. Clarke*

0000471



TITLE

XhoI digestion of 1.3K and 4.4K + gel analysis  
on 'late 5' for preparation of electro competent cells.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

17

From Page No. \_\_\_\_\_

Lisa continued analysis of mini-preps (see below)

Lisa is continuing with Bam HI digests of pII/2.25K.

Lisa is doing a maxi prep on the 1.3K clone.

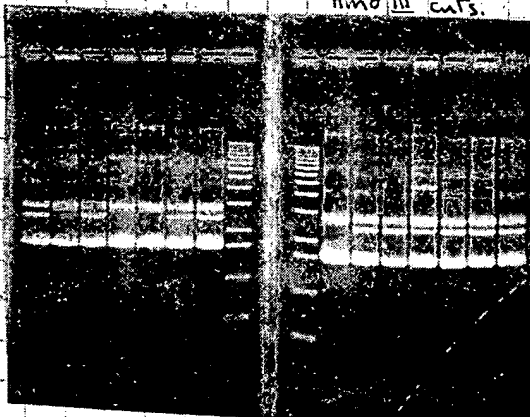
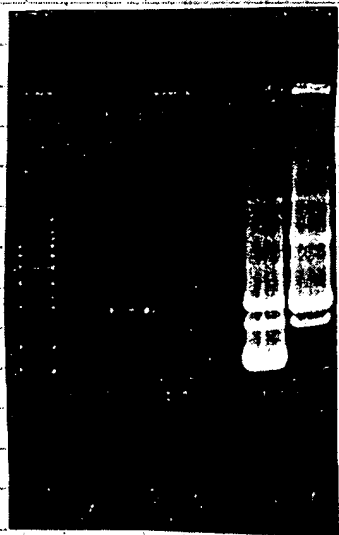
SphI cut  
pII/2.25K

Set up XhoI digestions on 1.3K and 4.4K DNAs.

1.3K in 30 $\mu$ l - 3 $\mu$ l Enz

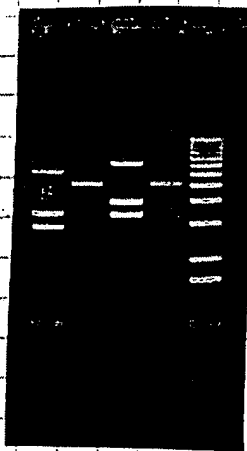
4.4K in 50 $\mu$ l - 5 $\mu$ l Enz ~ 4hrs at 37°C

Hind III cuts.



Analysis of remaining 1.5K + 2.25K  
in pII clones.  
None of the clones look as  
though they are what I need.

Ran samples from XbaI cut 1.3K, XbaI/Xho cut 1.3, SpeI cut 4.4K, and SpeI/XhoI  
cut 4.4K DNA.



Gel shows there is an additional SpeI site  
in the 4.4K - invalidating this approach.

Froze down hybridoma N°2.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Seiji W. Kibbi*

Date

Invented by

Date

Recorded by

*Warren K. Clarke*

0000473

From Page No. \_\_\_\_\_

Inoculated 1 litre of L-broth with one Late S' to prepare electrocompetent cells.

Grew to an OD<sub>600</sub> of 0.5 - then chilled in ice water for 15 mins

and proceeded with preparation according to George's protocol.

Froze down in ~100µl aliquots at -90°C in Box with

Devised a plan for uniting the 1.3K clone and the 4.4K clone without disturbing the orientation of the 1.3K clone with respect to the LacZ promoter etc.

Will use pluescript II SK (+) from stratagene.

(A)

1) Cut 4.4K with XhoI and BamHI → Gel purify 4.4K frag.

2) Bam/Xho cut (SK)<sup>phosphate</sup> and gel purify

3) Ligate both together and transform electrocompetent Late S' cells.

(B)

1) Cut 1.3K DNA with XhoI and KpnI → Gel purify

2) Cut 'A.3' above with KpnI and XhoI - phosphate → Gel purify

3) Ligate 1.3K into 'A3' <sup>Asp.</sup>

Checked in on hybridomas - froze down N°5 - stored N°2 with others at -80°C

Will freeze down a large batch tomorrow.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Serge W. Kibeli

Recorded by

David K. Clarker



From Page No. \_\_\_\_\_

Subcultured 27-32 from 6000 24-well plates into  
 25cm<sup>2</sup> flasks - froze down supers  
 Also froze down 10 more hybridomas from 25cm<sup>2</sup> flasks



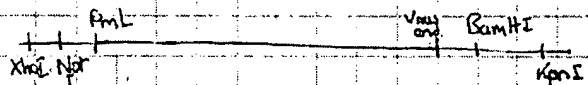
Lucas' gel of 1.3K DNA cut with EcoRI looks good.

A) The 2.25K fragment also looks good - Bam/Sph/HhaI cut.  
 It will be purified and ligated into SphI/BamHE cut <sup>phosphatase</sup> pET DNA.

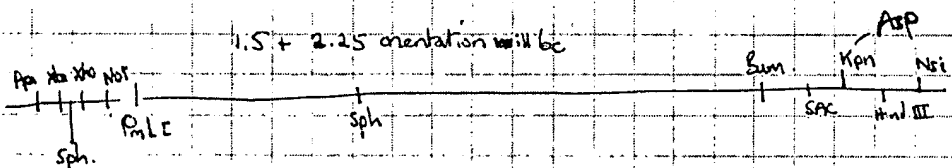
B) Then grown up and purified - then cut with ~~Bam~~ and  
 SphI and phosphorylated and gel purified.

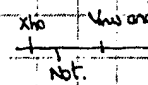
C) Then <sup>gel</sup>purify SphI/~~HhaI~~ cut (4) 1.5K DNA and ligate  
 into (B) above - first correct orientation by mini prep.

3K orientation is



1.5 + 2.25 orientation will be



① Can flip the 3K to give  then ligate the Pml-XhoI frag into Pml/XhoI cut  
 (1.5 + 2.25K)

OR

② Transfer the Bam/XhoI frag of 3K clone into BamHE cut  
 Bluescript II (SK+) then ligate in Pml/KpnI cut (1.5 + 2.25) into  
 pmlI/KpnI 3K + Bluescript <sup>phosphatase</sup> <sub>Ap</sub>

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Sege W. Keller*

Date

Invented by

Recorded by

*David K. Clatke*

Date

From Page No. \_\_\_\_\_

Prepared 300ml of L-Agar, Amp<sup>r</sup> and poured plates. Added IPTG and X-gal  
for colour screening

Carried out electrotransformation of Late 'S' cells using log of plasmid in  
~ 30µl of competent cells - pCII DNA, 4.4K DNA, 1.3K DNA.

plated 200µl of 1ml for each x 2.

Helped Lisa with gel purification of 2.25K and pCII DNAs which had been  
cut with SphI and BamHI. Phenol chloroform extracted and precipitated with  
1µl of glycogen.

Checked in on hybridoma - noted those which needed to be fed, sub-cultured etc.

as planned out Bluescript/4.4K digestions - will use 5µg of each in 50µl  
vol. with 5µl enzyme ~ 3hrs at 37°C

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Sgt. W. Kimbrell

Recorded by

David K. Clarke.

Xho I digestion of 4.4K and SK<sup>+</sup>(Bluescript) DNAs

Project No. \_\_\_\_\_

TITLE: Subculture of hybridoma cells.

Book No. \_\_\_\_\_

21

From Page No. \_\_\_\_\_

Checked transformants - there are quite a large number of transformants on all three plates; most on the pSV DNA plate - least on the 4.4K plate indicating that the efficiency may be size related. Will pick 4 colonies from each of 4.4K and 1.8K for mini preps to check plasmid integrity.

Set up Xho I digests of both 4.4K and Bluescript DNAs. in 50  $\mu$ l using 5  $\mu$ g of DNA and 5  $\mu$ l of enzyme (~100u) 1-15pm  $\rightarrow$  4-15pm.

checked hybridomas - froze down those which were well grown in 25cm<sup>2</sup>

flasks - sub-cultured hybridomas from 24-well plates into 25cm<sup>2</sup> flasks.  
(total of 5 - 33 to 37)

phenol chloroform extracted and EtOH ppt'd Xho I digests - saved 1/50<sup>th</sup> of each for gel analysis.

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Serge W. Kurbat*

Recorded by

*David R. Clarke.*

000477

From Page No. \_\_\_\_\_

Set up BamHI digestions of XhoI digested  $SV^{+}$  and 4.4K DNAs  
- in 50 $\mu$ l with 5 $\mu$ l of enzyme (100u) for 3hrs at 37 $^{\circ}$ C.

Meanwhile ran a gel to analyze XhoI digests of  $SV^{+}$  and 4.4K  
from yesterday.

Checked in on hybridomas - froze down another batch.

Checked gel of XhoI cut  $SV^{+}$  and 4.4K - Have run out of film - but  
digest looked good with both DNAs linearized and giving the expected bands.  
Thankfully!

Phenol chloroform extracted  $SV^{+}$  Bam/Xho cut DNA - EtOH pptd.

Saved 1 $\mu$ l from 4.4K/ $SV^{+}$  Bam digests for gel analysis.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Sup. W. Kibaki*

Recorded by

*David R. Clarke*

Phosphatase of SK<sup>+</sup> vector.  
TITLE Gel analysis of XhoI/Bam Cut SK<sup>+</sup> and 4.4K DNAs.

Project No. \_\_\_\_\_  
Book No. \_\_\_\_\_

23

From Page No. \_\_\_\_\_

Set up phosphatase rxn on SK<sup>+</sup> XhoI/Bam HI cut DNA. In 50 $\mu$ l

with 2 + 2.5 $\mu$ l of Enzyme over a ~1hr incubation at 37 $^{\circ}$ C. Then  
froze down prior to gel purification tomorrow along with Bam/Xho 4.4K DNA

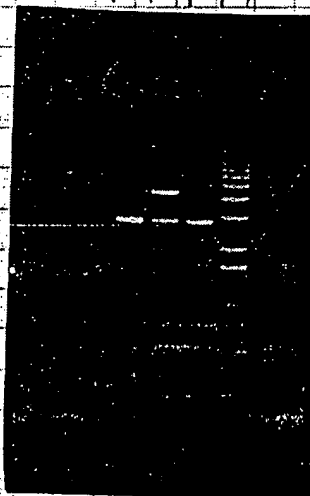
Prepared a 1% agarose gel for analysis of Bam/Xho Digests of SK<sup>+</sup> and 4.4K.  
(150 $\mu$ l of rxn)

Also ran Livas Double Digests SphI/Bam HI of gel purified DNA to  
quantitate (1 $\mu$ l of 20 $\mu$ l loaded for each)

Checked in on hybridomas - will need to subculture some from 24 well plates into

25cm<sup>2</sup> flasks; will also need to freeze down one or two perhaps.

2.25 Sph/Bam  
- put Sph/Bam  
4.4K Xho/Bam  
SK<sup>+</sup> Xho/Bam HI  
- 1KB ladder



2.25K has migrated a little unusually  
and is in low abundance (~10ng/ $\mu$ l).  
Will rerun a gel with 5 $\mu$ l of sample

Will get Lisa to ligate 2 $\mu$ l vector  
with 1 $\mu$ l insert 2.25K

SK<sup>+</sup> and 4.4K look fine

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

*Steve W. Kuhl*

Date

Invented by

Date

Recorded by

*David K. Clarke*

0000479

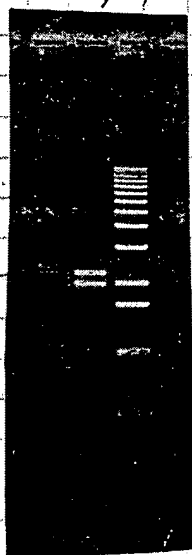
From Page No. \_\_\_\_\_

Run a 1% agarose gel to recheck the 2.25K gel purified fragment

Also run a 1% agarose gel to purify the 4.4K and 5K+ DNA (Xho/Bam digested).

Electroeluted, phenol/chloroform extracted and EtOH ppt<sup>d</sup> (with 1 $\mu$ l of glycogen).

Stored away previously frozen hybridomas and replaced Isopropanol in cell-freezing containers.

Purified 2.25K DNA.  
1KB Ladder.Froze down two more  
Hybridoma cell lines.Also passaged hybridomas  
#3, 38, 39, 40 into  
25cm<sup>2</sup> flasks.2.25K  
band still  
looks fine.And further split remaining  
24 well plate cells into  
mirrored wells to create  
more even monolayers.There is a 2<sup>nd</sup> smaller DNA species in the  
2.25K (purified DNA); this was most  
probably generated through nicking  
activity of HhaI followed by  
phenol/chloroform extraction (acidic; may  
have resulted in some breakage.)Lisa went ahead with the ligation + Con.  
5 $\mu$ l 2.25K DNA  
2 $\mu$ l Vector pCII  
2 $\mu$ l x10 buffer.  
1 $\mu$ l Enz.  
H<sub>2</sub>O to 20 $\mu$ l. O/N R temp.

Lisa set up O/N Minis for Electroporated transformation with 4.4K, 1.3K and pCII.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Invented by

Date

Serge W. Kurbali

Recorded by

David K. Blasche

Gel analysis of gel purified 4.4K and SK<sup>+</sup> DNA.  
o/n ligations of SK<sup>+</sup> and 4.4K DNA.

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

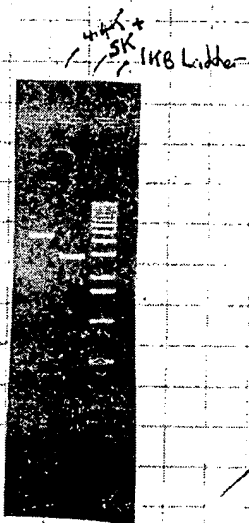
25

TITLE \_\_\_\_\_

From Page No. \_\_\_\_\_

Prepared a 1% agarose gel and loaded 1 $\mu$ l of 4.4K (gel purified) and SK<sup>+</sup> DNA (gel purified) - of 20 $\mu$ l total for each.

Lisa went ahead with the mini prep DNA extractions of 4.4K, 1.3K and pcII (Electroporation clones) - also set up EcoRI digests of these DNAs.



Good both the SK<sup>+</sup> and 4.4K gel purified DNAs look good and are abundant; ~100ng  $\mu$ l<sup>-1</sup>

Will ligate 4 $\mu$ l SK<sup>+</sup> with 3 $\mu$ l 4.4K o/n at R.Temp.

3 $\mu$ l SK.

4 $\mu$ l 4.4K

2 $\mu$ l x 10

+ CON SK<sup>+</sup> only.

10 $\mu$ l H<sub>2</sub>O.

1 $\mu$ l Enz.

20 $\mu$ l.

Checked in on Hybridomas - froze down one more #37

Stored Lisa's EcoRI digests of mini-preps (4.4K, 1.3K, pcII) at -20°C

Witnessed & Understood by me,

*Sage W. Kiehl*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

*David K. Clarke*

To Page No. \_\_\_\_\_

000481

From Page No. \_\_\_\_\_

Prepared 300ml L-Agar - for transformations - will use both electrocompetent and 'SURE' cells to get a comparative transformation efficiency

Spin down EtoII p.t. ligations - rinsed and resuspended in 6µl H<sub>2</sub>O  
Used 2µl to transform 'SURE' cells (from Con. + Test) 3-80 plate out.

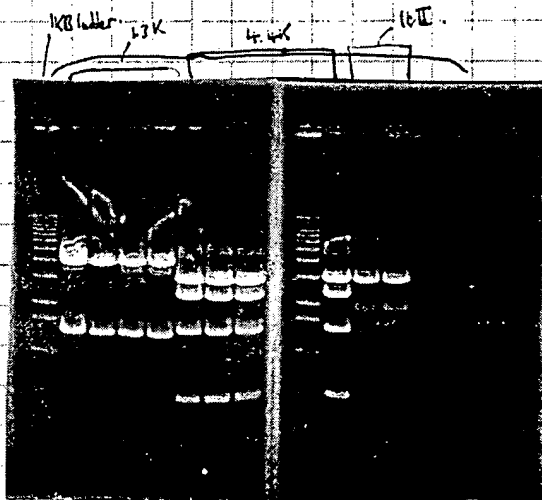
Also used 2µl to transform Electrocompetent cells (Late S<sub>2</sub>) 3-45 plate out.

ORDERED

'Sure' cells and -str. hygiene.

'SAP' - USB

see reference  
binds #074A



Gel showing Mini-preps  
from Electroporated  
1.3K, 4.4K, Full. It  
looks good; all the  
plasmids are amplified  
without significant  
modification.

Checked in on hybridomas - froze down # 34 and 35

~~Concerned the wisdom of putting all 8 fine gene segments on one plasmid~~  
~~by a 4.4K promoter and a 4.4K promoter. Could then transfer/electroporate in~~  
~~large plasmid containing all 8 gene segments. Could also transfer in another plasmid~~  
~~expressing support (helper) proteins. (e.g. T4 polymerase)~~  
~~Then use vaccinia to infect the cells. (e.g. T4 polymerase)~~

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzie W. Kibbe

Recorded by

David K. Clarke.

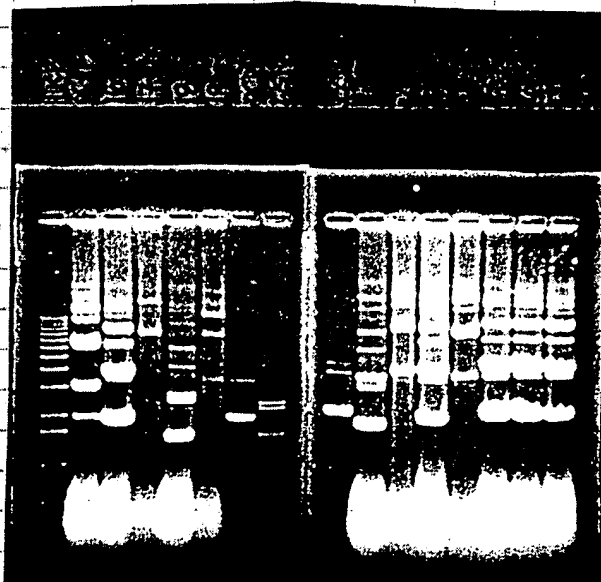


TITLE

Book No. \_\_\_\_\_

27

From Page No. ....



Will phenol chloroform extract - ppt and digest with Hind III

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

Recorded by  
David K. Clarke

000483

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE \_\_\_\_\_

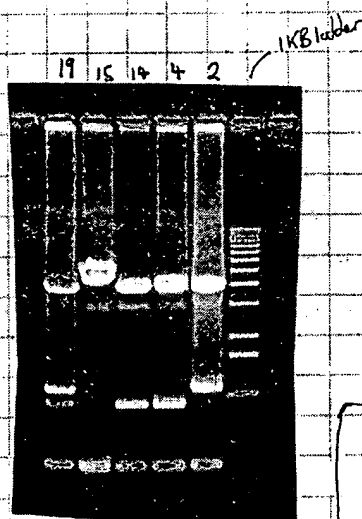
XhoI digestion of Asp718/KpnI cut 1.3K DNA.  
 HindIII digestion of Lysai minis - pot. 2.25K clones.  
 on minis of pot. 4.4K clones in SKT.

From Page No. \_\_\_\_\_

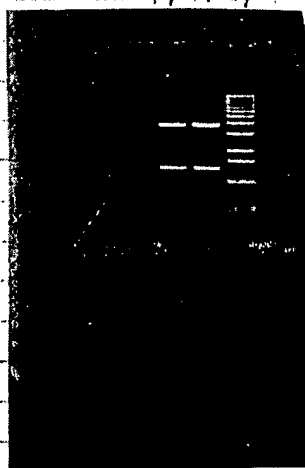
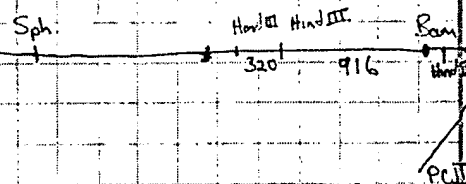
Set up XhoI digestions on both KpnI and Asp718 digested 1.3K DNA.  
 - in 50 $\mu$ l with 5 $\mu$ l of enzyme for 3-4 hrs (~3-45pm off)

Also phenol chloroform extracted Lysai potential 2.25K minis - EtOH.  
 ppt'd at -70°C for 30mins the set up HindIII digestions in 20 $\mu$ l with 1.5 $\mu$ l enzyme  
 and 2 $\mu$ l RNase A (off at 3-15pm)

Meanwhile checked hybridomas. froze down 4 more hybridomas



Gel shows that 4 and 14 are the ones I want (i.e. 2.25K in pelt).  
 Will set up O/Ns for Maxi preps



This gel indicates both  
 digests have worked well,  
 and indicate there is about  
 2-3 $\mu$ g of the 1.3K insert

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

Suzanne W. Kimble

Recorded by

David K. Clarke

From Page No. \_\_\_\_\_

Carried out mini prep DNA extraction on ten potential 4.4K clones (SURE)

Lisa did Electroporated clones. Will do EcoRE digestions on these DNAs

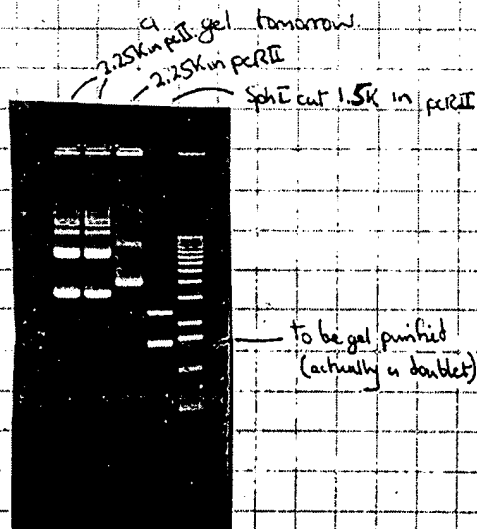
later. - Run 1% Agarose gels to analyse digests.

Prepared a 1% agarose gel and run out Asp718/Xho cut 1.5K DNA for gel purification.

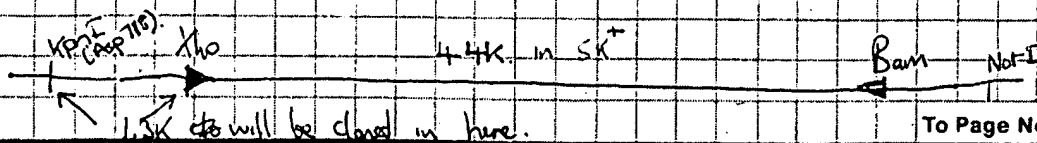
Lisa is doing maxi preps on 4 and 14.

Set up SphI digestion of 1.5K DNA in 50µl (used 10µl of 1.5K(4) stock).  
 Used SphI Enzyme for 3-4 hrs - run a 1µl sample after ~2 hrs  
 to check digestion efficiency.

Electro eluted the 1.5K DNA - phenol extracted and EtOH ppt'd - Will check 1/20 on



2, 4, 7, and 9 look as though they are correct. Grew up 2 and 4 as gms maxi cultures



Witnessed & Understood by me,

Date

Invented by

Date

Sage W. Kurble

Recorded by

David K. Clarke

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Gel analysis of purified 1.3K DNA (Asp/Xho cut).

Hind III digestion of 2.25K maxis 4 + 14.

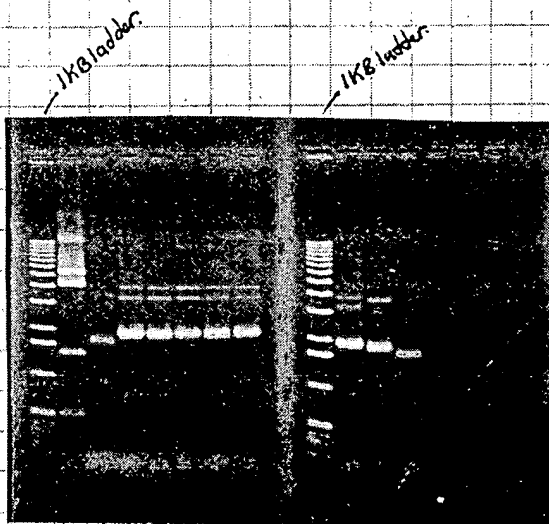
Gel purification of Sph 1.5K and Sph 2.25K in pCII.

From Page No. \_\_\_\_\_

Prepared a 1% agarose gel for 1.3K DNA analysis. Spun down 1.3K DNA  
mixed-dried and redissolved in 20  $\mu$ l  $H_2O$ . Looked at 1  $\mu$ l on a gel.

Set up Hind III digestions on 2.25K DNA (2  $\mu$ l DNA) in 20  $\mu$ l - 1  $\mu$ l Enz. ~ 2 hrs  
37°C.  
ran out on a 1% agarose gel to check.

Lisa is doing 4.4K in SK<sup>+</sup> 2, and 4. (Maxi preps) - freezing down 4 and 4 (2.25K  
in pCII).



Gel shows EcoRI digests  
of Electroporated 4.4K  
into SK<sup>+</sup> vector. None of  
the clones appear to be  
correct.

Froze down more hybridomas. Will sub-culture some into 25cm<sup>2</sup> Flasks.

Spun down maxi-prep 4.4K in SK<sup>+</sup> - looked at 1  $\mu$ l of each on a gel.

Set up EcoRI digests on 1  $\mu$ l to recheck.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

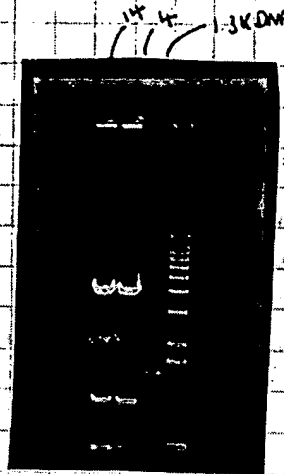
Serge W. Kimbali

Recorded by

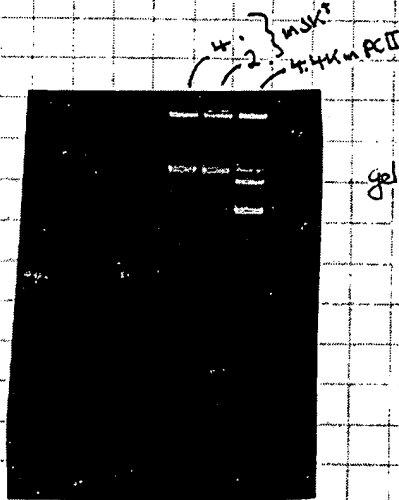
David K. Clarke.

TITLE \_\_\_\_\_

From Page No. 30



Gel shows gel purified 1.3K DNA ~ 50ng  $\mu$ l<sup>-1</sup>  
also shows Hind III digested 2.25K (4 and 14)  
They both look fine.



gel shows 2 and 4 (4.4K in skt) maxi  
preps - which are showing a very low  
yield. Will repeat maxis with ⑦ and ⑨

Decided to set up O/N Maxis of  
⑦ and ⑨ to try and get an improved  
yield.

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

*Suzanne W. Kurbale*

Date \_\_\_\_\_

Invented by \_\_\_\_\_

Date \_\_\_\_\_

Recorded by \_\_\_\_\_

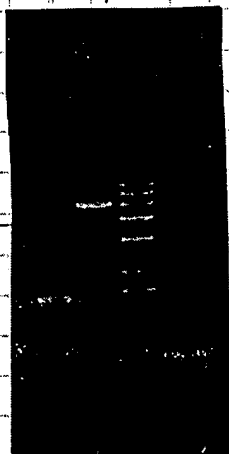
*David K. Clarke*

From Page No. \_\_\_\_\_

Prepared a 1% agarose gel for analysis of gel purified  $\text{SphI}$  cut 2.25K in  $\text{Pst}$ , and  
 $\text{SphI}$  fragment of 1.5K DNA. There is  $\sim 25\text{ng}$  of 1.5K DNA  $\mu\text{L}^{-1}$  (half is  
 due to the presence of some vector DNA).

There is  $\sim 150\text{ng}$   $\mu\text{L}^{-1}$  for 2.25K DNA. - Although there is some contaminating  
 uncut plasmid too.

1.5K DNA  
 2.25K  $\text{Pst}$



Redigested 2.25K uncut  
 with  $\text{SphI}$  ( $\sim 1\mu\text{g}$ ) in  
 30 $\mu\text{L}$  with 2 $\mu\text{L}$  of enzyme  
 to try and eliminate remaining  
 uncut DNA. - Will then phenol  
 extract - EtOH ppt and  
 phosphatase tomorrow.

Meanwhile prepared L-Agar  
 for restreaking clone banks - poured and  
 dried plates.

Will phosphatase 1 $\mu\text{g}$  of 2.25K DNA in  
 50 $\mu\text{L}$

8 $\mu\text{L}$  2.25K. 0.5 $\mu\text{L}$  2.25K

5 $\mu\text{L}$  X10

35 $\mu\text{L}$  H<sub>2</sub>O

2 $\mu\text{L}$  phosphatase

50 $\mu\text{L}$  -  $\sim 30\text{min}$  at 37°C

added another 1.5 $\mu\text{L}$  of phosphatase  
 and left at 37°C for  $\sim 40\text{min}$  longer

lisa set up ON ligation of 1.5K  
 into 2.25K in  $\text{Pst}$

1 $\mu\text{L}$  of 2.25K (It was  
 resuspended in 5 $\mu\text{L}$  H<sub>2</sub>O)  
 2 $\mu\text{L}$  1.5K DNA ( $\sim 50\text{ng}$ )

2 $\mu\text{L}$  X10 buffer

1 $\mu\text{L}$  Enzyme

14 $\mu\text{L}$  H<sub>2</sub>O

20 $\mu\text{L}$  ON at 37°C

Mucci preps of 4.4K (9 and 7)  
 did not work out.

Decided to colony purify

4.4K (Sure) #4

Restreaked entire clone bank

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me.

Date

Invented by

Date

Steve W. Kimbrell

Recorded by

Daniel K Clarke

Phosphatase of SphI redigested 2.25K in pcII (Previously cut and gel purified) Project No. \_\_\_\_\_  
TITLE O/N minis to colony purify 4.4K in SK<sup>+</sup> Book No. \_\_\_\_\_

From Page No. \_\_\_\_\_

Checked colony purified 4.4K in SK<sup>+</sup> (#4) - there are individual colonies which will be used to set up o/n mini preps.

Set up phosphatase rxn on Redigested (SphI) 2.25K in pcII.  
40  $\mu$ l final vol ( $\sim$  1  $\mu$ g of DNA)  
2  $\mu$ l phosphatase + 1  $\mu$ l after  $\sim$  40 mins at 37°C.  
(Recessed 5' ends will reduce efficiency.) phenol extracted and etoh. ppt'd with 2  $\mu$ l of glycogen.

Set up 8 o/n mini cultures of potential 4.4K Amp<sup>r</sup>. Will do minis tomorrow, along with EcoRE digests/gel analysis.

Stored clone bank plates at 4°C - will incubate some for a further 24 hrs to improve colony growth.

Lisa carried out transformations with o/n ligation. 1.5K + 2.25K in pcII DNA. She also did a vector (unligated) control to check for level of background due to unligated vector (in ligation mixture).

Witnessed & Understood by me,

*Lisa W. Kuhl*

Date

Invented by

Recorded by

*David K. Clarke*

Date

To Page No. \_\_\_\_\_

Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

TITLE

Mini prep analysis of colony purified 4.4K  
Gel analysis of redigested 2.25K, SAP<sup>+</sup> DNA in pCII.

From Page No. \_\_\_\_\_

Checked Lisa's clones 1.5K + 2.25K in pCII - there are many white colonies; however the controls are pale blue (may indicate some readthrough of the 2.25K insert)

Carried out mini preps for potential 4.4K clones in SK<sup>+</sup> vector.

20  $\mu$ l (of 50  $\mu$ l) of the final mini prep suspension in H<sub>2</sub>O was digested with EcoRI, and the products were analysed on a 1% agarose gel (The remaining DNA was frozen at -20°C) - may need to use it to transform 'late S'

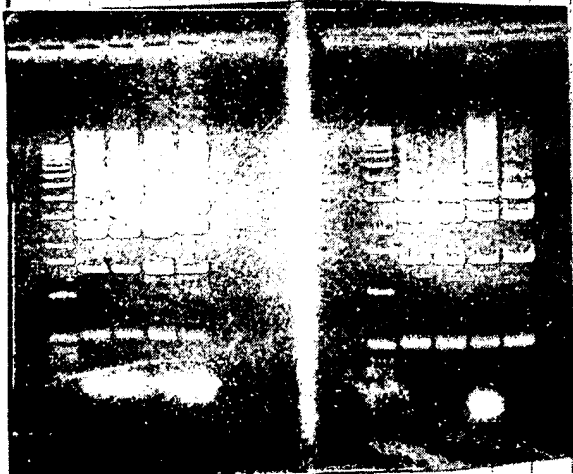
Spin down redigested (SphI) 2.25K DNA in pCII - resuspended in 10  $\mu$ l H<sub>2</sub>O and run 1  $\mu$ l on a gel to quantitate.

2.25K in pCII SphI cut, SAP<sup>+</sup>  
Redigested

All 8 clones look good - Lisa will grow up ① and ④ as Max's.



Conc = 100ng  $\mu$ l<sup>-1</sup>



← Gel shows 8 potential 4.4 clones in pCII - all look fine (they were all derived from original clone 4 (see page 29) Subcultured by hand into 4b, 47, 48

To Page No. \_\_\_\_\_

Witnessed &amp; Understood by me,

Date

Invented by

Date

*Serge W. Kurbule*

Recorded by

*David K. Clarke*



Discussion of progress with Lisa.  
 TITLE: Discussion of future direction with Kathy.

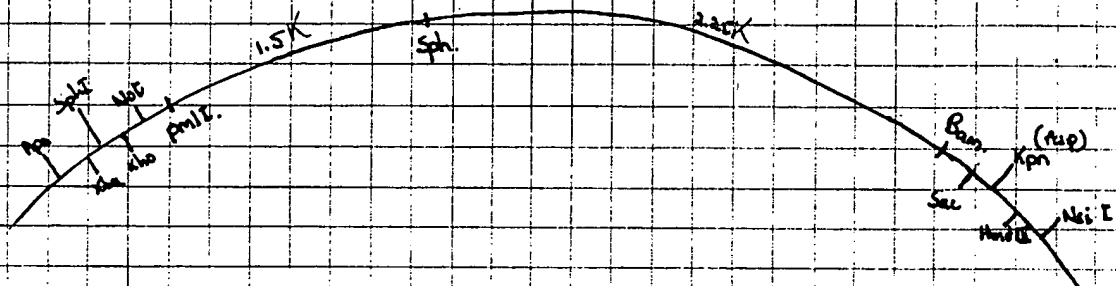
Project No. \_\_\_\_\_

Book No. \_\_\_\_\_

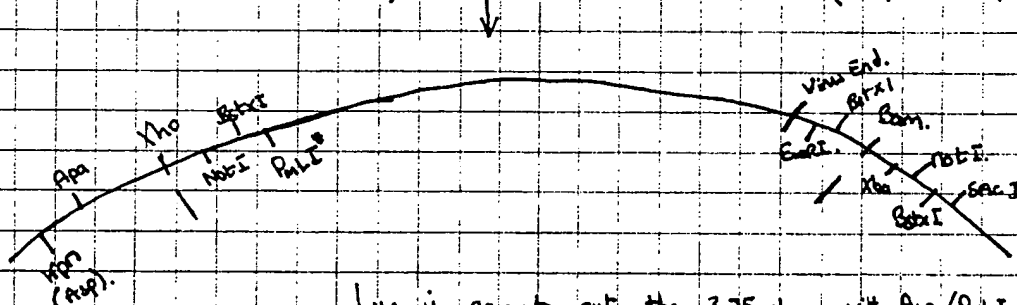
35

From Page No. \_\_\_\_\_

Lisa has done a fine job and has successfully linked the 1.5K and 2.25K clones to give a 3.75K clone in ~~SK+~~ <sup>PCNAII</sup> ~~SK+~~ <sup>PCNAII</sup>



The 2K clone (in PCNAII) has been transferred into SK+ (Bam/XhoI site)



Lisa is going to cut the 3.75 clone with Asp/PstI and gel purify the fragment and ligate it into Asp/PstI cut (3K in SK+)

Lisa is rechecking the PstI enzyme (the central supply stock did not cut)  
 (non supply). (The other stock of PstI is fine)

4.4K DNA could not be grown up in large amounts - will set up transform preps and

try to prepare DNA from them

To Page No. \_\_\_\_\_

Witnessed & Understood by me,

Date

Invented by

Date

*Steve W. Kelle*

Recorded by

*David K. Clarke*

000491